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Biparental Endowment of Endogenous Defensive Alkaloids in *Epilachna paenulata*

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Abstract Coccinellid beetles contain a variety of defensive alkaloids that render them unpalatable to predators. *Epilachna paenulata* (Coleoptera: Coccinellidae) is a South American ladybird beetle that feeds on plants of the Cucurbitaceae family. The defensive chemistry of *E. paenulata* has been characterized as a mixture of systemic piperidine, homotropane, and pyrrolidine alkaloids. Whole body extracts of adult beetles contain four major alkaloids: 2-(2′-oxopropyl)-6-methylpiperidine (**1**); 1-(6-methyl-2,3,4,5-tetrahydro-pyridin-2-yl)-propan-2-one (**2**); 1-methyl-9-azabicyclo[3.3.1]nonan-3-one (**3**); and 1-(2″-hydroxyethyl)-2-(12′-aminotridecyl)-pyrrolidine (**4**). Comparative studies of the defensive chemistry of eggs, larvae, pupae, and adults showed differences in alkaloid composition and concentration among life stages. While adults contained mainly the homotropane 1-methyl-9-azabicyclo[3.3.1]nonan-3-one (**3**), eggs showed the highest concentration of the piperidine 2-(2′-oxopropyl)-6-methylpiperidine (**1**). We studied the origin of this alkaloid in the eggs by feeding newly emerged, virgin adult beetles with [2-^{13}C]-labeled acetate, and by performing crosses between ^{13}C-fed and unlabeled males and females. GC-MS analysis of alkaloids from ^{13}C-fed males and females showed high incorporation of ^{13}C into the alkaloids, as evidenced from a 20–30% increase of isotopic peaks in diagnostic fragment ions, confirming the expected endogenous origin of these alkaloids. In addition, analyses of eggs from different crosses showed that labeled alkaloids from both parents are incorporated into eggs, indicating that *E. paenulata* males transfer alkaloids to the females at mating. Biparental endowment of chemical defenses into eggs has been shown previously in insects that acquire defensive compounds from dietary sources. To our knowledge, this is the first report of biparental egg endowment of endogenous defenses.

Keywords *Epilachna paenulata* · Nuptial gift · Biparental endowment · Egg defense · Coleoptera · Coccinellidae

Introduction

Ladybirds have been a remarkable source of novel structures, mainly alkaloids (Glisan King and Meinwald 1996) and polymeric nitrogen-containing substances (Schroeder et al. 1998a, b). Such compounds, which may have a clear defensive purpose (Eisner et al. 1986, 2002; Attygalle et al. 1993a, b; Glisan King and Meinwald 1996), include azaphenalenes, azabicyclo nonanes (homotropane), aliphatic and aromatic amines, pyrrolidine and piperidine alkaloids, azamacrolides and macrocyclic polyamines. In most cases, coccinellid alkaloids are of endogenous origin (Attygalle et al. 1994; Laurent et al. 2001, 2002), although a few studies have reported ladybird species that sequester alkaloids from their food source (Witte et al. 1990; Pasteels 2007).

Epilachna paenulata Germar 1824 (Coleoptera: Coccinellidae) is a phytophagous South American ladybird beetle that feeds on the leaves of cucurbits. The adults exhibit defensive capacity against generalist predators, due to the presence of systemic alkaloids that have been characterized

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as a mixture of piperidine, homotropane, and pyrrolidine alkaloids (Scheme 1). Specifically, adult beetles contain four major alkaloids: 2-(2′-oxopropyl)-6-methylpiperidine (**1**); 1-(6-Methyl-2,3,4,5-tetrahydro-pyridin-2-yl)-propan-2-one (**2**); 1-methyl-9-azabicyclo[3.3.1]nonan-3-one (**3**); and 1-(2″-hydroxyethyl)-2-(12′-aminotridecyl)-pyrrolidine (**4**) (Camarano et al. 2006). These alkaloids are present in all life stages of the insect, albeit showing qualitative and quantitative variations among developmental stages (Camarano et al. 2006).

We have shown previously that the highest alkaloid concentration in *E. paenulata* is found in the eggs. Indeed, the concentration of the main egg alkaloid, piperidine **1**, is higher in the eggs than in the females that laid those eggs (Camarano et al. 2006), thus suggesting some mechanism of preferential endowment of alkaloid from females to eggs, or an additional source of alkaloid that finds its way into the eggs. We investigated the latter hypothesis. Specifically, we asked whether males transfer part of their alkaloids to the female, and if so, whether these alkaloids are found in the eggs.

Methods and Materials

Insects E. paenulata adults were obtained from a laboratory colony regularly maintained on squash plants (*Cucurbita* sp.), under controlled conditions of temperature (23±2°C) and a photoperiod L:D 16:8 h. For the initial settlement of the colony, individuals were collected on squash plants at organic farms near Montevideo, and new field-collected individuals were added every year. The different life stages were maintained separately in screened cages (30×30×30 cm), and 9 to 12 food plants (3-wk-old) were replaced every 3–4 days (Camarano et al. 2006).

Alkaloid Labeling Incorporation of ^{13}C labels to the alkaloids was done by feeding the adult beetles with squash leaves treated topically with [2-^{13}C]-sodium acetate (40 mg/ml in MeOH).

To analyze a pool of individuals (N=25), complete squash leaves were treated with 3.2 μg of labeled acetate/beetle/day. Beetles were allowed to feed for 5 days and then frozen for alkaloid analysis.

For hemolymph analyses, 80 μl of the same solution were applied on squash leaf disks (2.5 cm diam), and the disks were offered to newly emerged adults (N=8) caged in individual containers (4 cm diameter×8 cm height). Leaf disks were placed on agar (2%) to avoid dehydration and replaced daily. On day 5, the beetles were gently disturbed with forceps to trigger the reflex bleeding reaction that is typical of insects with systemic defenses, and the hemolymph was collected in glass capillaries (5 μl, Drummond Sci. Co.) for alkaloid analysis (González et al. 1999a; Camarano et al. 2006; Camarano 2008). The same protocol was followed to label the alkaloids in male or female beetles used for mating experiments and egg collection.

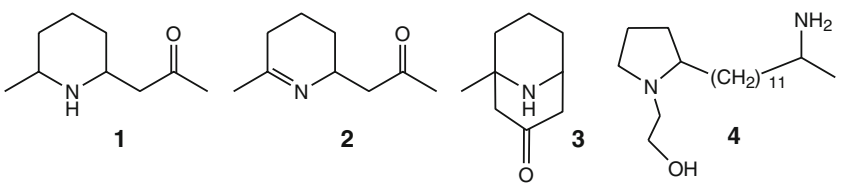
Control experiments were run under identical conditions for both labeling procedures, treating the leaves or leaf discs with methanol. Control and ^{13}C-fed beetles were all kept under the same environmental conditions described above.

Mating and Egg Collection Horizontal (male to female) and vertical (female to egg) alkaloid flow were studied by setting different mating pairs, in which one of the parent beetles had been fed with ^{13}C acetate (hereafter indicated with an asterisk: ♀* or ♂*).

To study male alkaloid transfer, eight virgin control females (♀) were placed individually with virgin ^{13}C-fed males (♂*). To determine if copula had taken place, the position of the beetles was recorded on video (Panasonic AG-DP200E Camera, recording 1 s every 10 min). After mating, the male was removed, and hemolymph was collected from the female 24 h later. Females were then allowed to lay eggs in the same containers (see next paragraph).

The vertical flow of alkaloids was studied similarly by collecting eggs from three groups of mating pairs: ♀* × ♂ (N=4), ♀ × ♂* (N=4), and ♀ × ♂ (N=2, control).

Alkaloid Extraction Three procedures for alkaloid extraction were carried out, depending on the matrix and sample size: (1) 25 pooled adults were killed by freezing and extracted under stirring with methanol (3 h); the extract was filtered, dried under vacuum, re-suspended in HCl (0.2 M), and washed with hexane (3×15 ml). The pH of the remaining aqueous phase was increased to 10 (NaOH, 1 M), and the alkaloids were extracted with methylene



Scheme 1 Systemic alkaloids identified in *E. paenulata*

chloride (3×15 ml). This final extract was concentrated under vacuum, at room temperature; (2) alkaloids from the hemolymph were extracted by suspending freshly collected hemolymph in 200 µl of ammonium hydroxide (pH 8) and extracting the alkaloids with methylene chloride (3× 150 µl); (3) alkaloids from the eggs were extracted with methanol (1 ml) at room temperature (24 h). Eggs were crushed to facilitate the extraction, and the solid remains were separated by centrifugation (14,000 rpm, 10 min). The supernatant solution was taken to pH 8 (NaOH), and extracted with methylene chloride (3×150 µl). In the two latter procedures, the joined methylene chloride extractions were concentrated to ca. 100 µl under N_2.

Analytical Procedures To determine ^{13}C incorporation into *E. paenulata* alkaloids, the extracts were analyzed by GC-MS as follows: extracts from pooled adults were analyzed by using an HP 5791 instrument equipped with an Elite-5 column (30 m; 0,32 mm), splitless injection in CH_2Cl_2, and a temperature program starting at 60°C (4 min) to 300°C (8 min) at 10°C/min. Injector and interphase temperature were 300°C, and total ion current detection mode was used. In the case of egg and haemolymph extracts, GC-MS analyses were performed on a Shimadzu QP 5050 or a Shimadzu QP 2010 [Carbowax 20M, 25 m, 0.32 mm; splitless injection in CH_2CI_2, 60ºC (6 min) - 204ºC (20 min) at 10ºC/min. Injector and interphase temperature: 250ºC]. Alkaloids (**1**, **2**, and **3**), where incorporation was studied, were identified previously on the basis of NMR and/or mass spectral data (Camarano et al. 2006). MS data are included in Table 1, Supplementary material. To enhance sensitivity, detection of alkaloids in the egg extracts was done by single ion monitoring (SIM) of the characteristic ions, specifically choosing the following peak clusters: 98-99-100-101-102-103 (characteristic of alkaloid **1**), 110-111-112-113-114-115-116 (characteristic of alkaloids **2** and **3**), 153-154-155-156-157-158-159-160 [cluster including M+ of **1** (*m/z*=155) and **2** and **3** (*m/z*=153)] (Camarano 2008). A validation process comparing mass spectra acquisition by two modes, total ion current and single ion monitoring, was carried out to ensure that acquisition by SIM had no effect on the data (Supplementary material Figs. 1 and 2).

Calculations of ^{13}C-enrichment of alkaloids were performed with freeware developed by the National Institutes of Health, US (available on line at http://sx102a.niddk.nih.gov/iso.html, last accessed in 2007, Hess et al. 2002). The program compares the relative intensities of all ion peaks within an ion cluster, for enriched and control samples, and calculates the enrichment as percent MPE (mole percentage excess) of the stable isotope related to its natural abundance, as well as the maximum number of labels incorporated.

Results

[2-^{13}C]-Acetate Incorporation into E. paenulata Alkaloids The analysis of alkaloid extracts from pooled adults (*N*=25) showed only the homotropane **3**. Analysis of the corresponding peak clusters (*m/z* 110 and 153) showed an incorporation of ^{13}C from acetate into this alkaloid, with a MPE of 22%.

In hemolymph samples, despite their low concentration, alkaloids **1**, **2**, and **3** were detected by GC-MS analyses in all the samples (e.g., alkaloid **3**, Fig. 1). The relative intensities within different ion clusters showed that *E. paenulata* incorporated labeled acetate into the three defensive alkaloids, with MPE of 20% to 30% (Table 1, Fig. 1). Noteworthy, this methodology allows the follow-up of ^{13}C incorporation within an individual through time, since hemolymph extraction does not injure the insect.

Alkaloid Egg Endowment (Vertical Flow) GC-MS analyses of egg samples showed detectable amounts of alkaloids **1** and **3** (SIM detection). Extracts of eggs laid by females from ♀* × ♂ mating pairs evidenced ^{13}C incorporation (Table 2) with up to five labels in alkaloid **3**, indicating that females transfer their own alkaloids to the eggs. Interestingly, the analyses of eggs laid by control females mated to ^{13}C-fed males (♀ × ♂*) also showed labeled alkaloids, indicating that male alkaloids are also transferred to the eggs they sire (Table 2, Fig. 2).

Male Nuptial Gift (Horizontal Flow) The existence of an alkaloidal nuptial gift from males to females at mating can be inferred from the fact that male alkaloid finds its way into the eggs. We also studied the fate of the male alkaloids in the females, by analyzing the hemolymph of mated females from ♀ × ♂* mating pairs. The main alkaloids in these hemolymph samples were **2** and **3**, but no evidence of ^{13}C labeling was found, suggesting that the male alkaloids do not pass the systemic circulation in the female, but rather remain confined to the female reproductive tract.

Discussion

The three major alkaloids (**1-3**) in *E. paenulata* showed incorporation of [2-^{13}C]-acetate when fed with squash leaves treated with the labeled precursor. These results confirm the notion that defensive alkaloids in this beetle are endogenous, as it is the case in other coccinellid species (Attygalle et al. 1993b, 1994, 1999; Glisan King and Meinwald 1996; Farmer et al. 1997; Radford et al. 1997; Schroeder et al. 1998a, b; Braekman et al. 1999; Laurent et

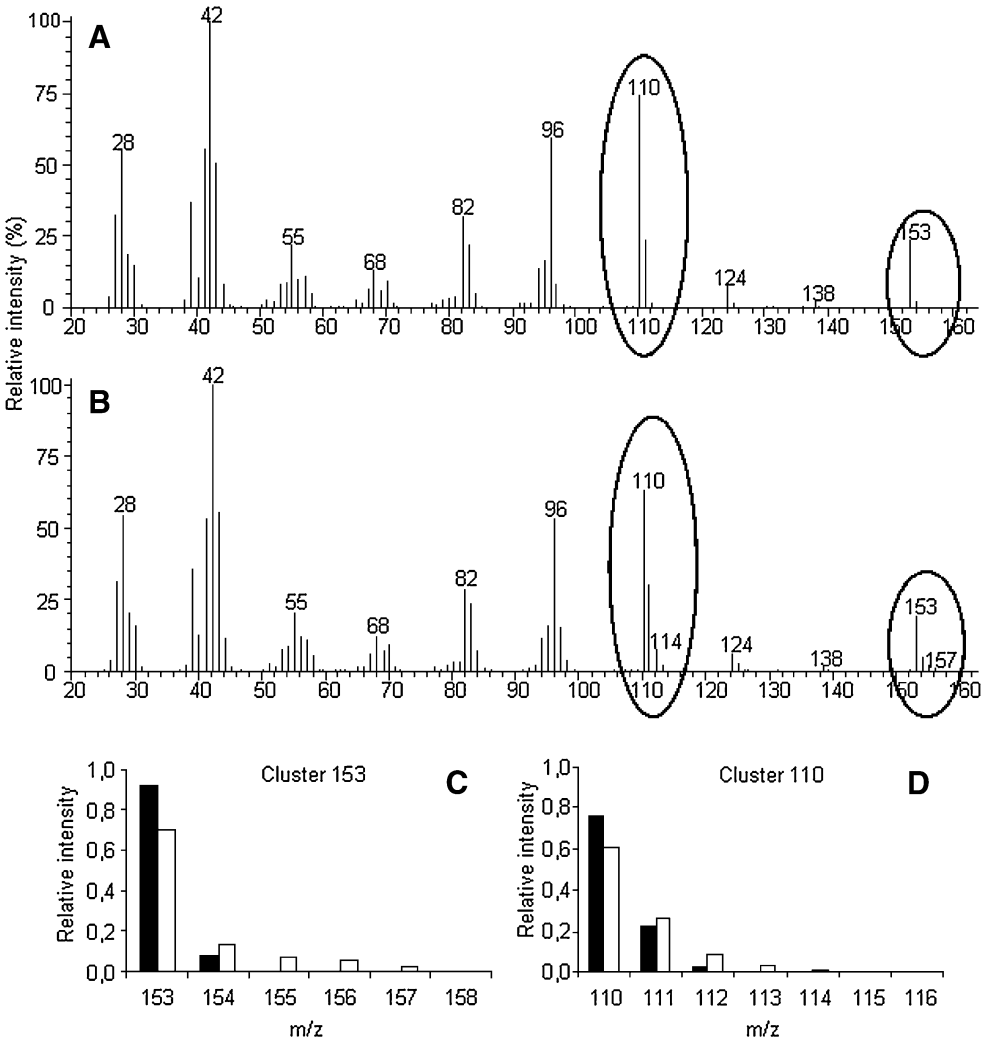


Fig. 1 MS trace of alkaloid **3** from hemolymph samples of *E. paenulata* individuals fed on normal diet (**a**) and on [2-^{13}C]-sodium acetate enriched diet (**b**). Two characteristic ion clusters show evidence for ^{13}C incorporation, given the relative increase of ions up to *M*+4. The relative intensities are presented in normalized form in **c** (*m/z* 153 cluster) and **d** *m/z* 110 cluster (*filled squares* control; *empty squares* ^{13}C-enriched samples) (TIC detection)

al. 2001, 2002). When using SIM detection, our data show that the alkaloids incorporated up to five acetate units (Table 2), which is in accordance with the polyacetate biosynthetic pathway that has been proposed for coccinellid alkaloids (Laurent et al. 2001). Preliminary experiments also showed weak incorporation of [18-D_3]-stearic acid into *E. paenulata*'s piperidine **2** (data not shown), suggesting a fatty acid-derived pathway. This is in agreement with earlier findings by Laurent et al. (2002), who demonstrated a fatty-acid biosynthetic pathway for the alkaloids adaline and coccinelline in *Adalia 2-punctata* and *Coccinella 7-punctata*, respectively. In plants, pyrrolidine and piperidine alkaloids are derived from amino acids such as ornithine and lysine, which cannot be ruled out from our data (Dewick 1997).

The incorporation of ^{13}C into the alkaloids allowed us to study the horizontal and vertical flow of chemical defenses in this beetle, since it made it possible to differentiate alkaloids from males and females. Eggs coming from one "labeled" parent, either female or male, showed alkaloid **1** and **3** labeled with ^{13}C (Table 2). Although egg protection by female defensive compounds is expected, our data show that males also can protect the eggs that they sire, by transferring part of their alkaloidal load to the females at mating. Since the males were removed from the mating container several days before the eggs were laid, the male must transfer the alkaloids to the female, and she then transfers the male's and her own alkaloids to the eggs.

Table 1 Enrichment of *E. paenulata* alkaloids in hemolymph samples from adults fed with [2-^{13}C]-sodium acetate

Alkaloid	*m/z*	Max. number of labels	Percent MPE[a] (mole percentage excess)
1[b]	98	1	26±5
2	110	2	26±9
	153	2	24±10
3	110	3	23±7
	153	4	24±7

[a] Data are shown as mean±SD (*N*=8) of the % MPE
[b] For alkaloid **1**, results are presented for ion cluster *m/z* 98, due to the low intensity of the M^+ cluster (*m/z* 155)

Table 2 Enrichment and maximum number of labels incorporated to alkaloids **1** and **3** in *E. paenulata* egg samples from different mating pairs

Alkaloid	*m/z* cluster	Origin of egg sample			
		♀* x ♂		♀ x ♂*	
		Max. number of labels	Percent MPE	Max. number of labels	Percent MPE
1	98	3	16±4	3	18±7
3	110	3	28±5	3	23±6
	153	5	23±5	3	20±5

Results are shown as mean±SD (N=4 for both mating types)
* Denotes individuals fed on [2-^{13}C]-sodium acetate enriched diet

Insect eggs often contain chemical defenses, and these toxins are provided either by the female, the male, or both sires (Hinton 1981; Hilker 1994). Males of several insect species pass a nuptial gift to the female at mating (Vahed 1998, 2007), and it is not uncommon that part of this gift is allocated to the eggs (Vahed 1998; Eisner et al. 2002). Biparental endowment of egg defenses has been demonstrated in a number of systems in which the defensive chemicals are acquired from the diet (Ferguson et al. 1985; Dussourd et al. 1988; Nishida and Fukami 1989; Blum 1992; González et al. 1999a; Hartmann 1999; Eisner et al. 2002). To our knowledge, however, biparental egg endowment of endogenous defenses, as shown here, has not been demonstrated before. In the case of cantharidin, a terpene anhydride produced de novo by some oedemerid and meloid beetles, the situation varies among species. In *Oedemera femorata* (Coleoptera: Oedemeridae), both sexes produce cantharidin, but females produce higher amounts than males. Transfer of cantharidin from males to females at mating was not found in this species, and hence, cantharidin in the eggs appears to originate solely from females (Carrel et al. 1986; Holz et al. 1994). In members of Meloidae, such as *Lytta vesicatoria*, the females are unable to produce their own cantharidin, and they receive it as a nuptial gift from males at mating. Females then bestow part of this cantharidin gift into the eggs (Meyer et al. 1968; Schlatter et al. 1968; Sierra et al. 1976; Eisner et al. 2002). Conversely, in the case of *Epicauta funebris*, only adult males keep the larval capability of biosynthesizing cantharidin, and although there is seminal transmission of cantharidin, it is not transferred into eggs, but rather kept by the females, likely for self-protection (McCormick and Carrel 1987; Carrel et al. 1993).

In *E. paenulata*, the presence of male alkaloid in the eggs indicates a nuptial gift, but the pathway by which this gift reaches the eggs remains unknown, since we did not detect male-labeled alkaloid in the hemolymph of the females they mated with. This result suggests that most of the male alkaloid remains confined within the female reproductive tract, from where it is allocated into the eggs, probably as they are laid. Careful dissections of the female reproductive tract and analysis of its parts, in particular the spermatheca (Rossini et al. 2001; Nikbakhtzadeh et al. 2007), should provide insight about the fate of male alkaloids after seminal transfer. In other insects, defensive male nuptial gifts become systemic within the female body. In *Utetheisa ornatrix* (Lepidoptera: Tortricidae), for exam-

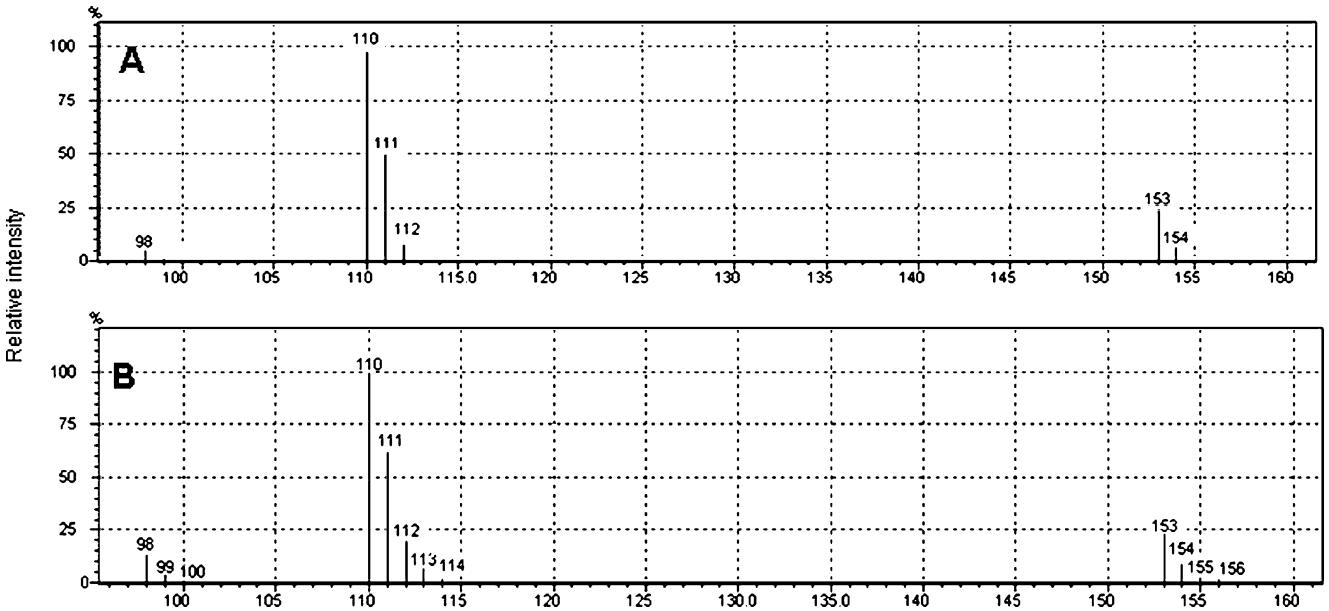


Fig. 2 MS traces of homotropane **3** from eggs laid by control *E. paenulata* females (**a**) and control *E. paenulata* females mated to ^{13}C-fed males (**b**) (SIM detection)

ple, females can use the pyrrolizidine alkaloids transferred by males both for their own defense and for the protection of the eggs (González et al. 1999b), since the alkaloids become systemic even before the mating pair uncouples.

The adaptive value of male egg protection in *E. paenulata* is open to question. The female load may be insufficient to properly protect the eggs against predators and/or microorganisms. Obtaining and testing eggs with different alkaloidal loads, below and above female levels, would be useful to test this hypothesis. If male defenses do make a difference for the fitness of the female, it opens the possibility for some overt or cryptic process of sexual selection that may favor males with larger alkaloid loads.

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Response of the Egg Parasitoids *Trissolcus basalis* and *Telenomus podisi* to Compounds from Defensive Secretions of Stink Bugs

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Abstract We tested the hypotheses that host-searching behavior of the egg parasitoids *Telenomus podisi* and *Trissolcus basalis* may be differentially influenced by the different blends of volatiles released from the metathoracic glands of adult stink bug host species. We further studied whether such a differential response is due to different individual components of these glands and whether these responses reflect host preferences. Y-tube olfactometer bioassays were carried out with crude extracts of metathoracic glands of five different host species of neotropical stink bugs. Additionally, we tested the parasitoids' responses to synthetic standards of individual compounds identified in these stink bug glands. Results showed that females of *T. basalis* and *T. podisi* responded differentially to crude gland extracts of the different species of host stink bugs and to the compounds tested. The parasitoid *T. basalis* showed a positive taxic behavior to *Nezara viridula* methathoracxic gland extracts of a host species preferred in the field, i.e., *N. viridula.* Furthermore, *T. basalis* responded positively to 4-oxo-(*E*)-2-hexenal and (*E*)-2-decenal, two components of *N. viridula* glandular secretion. Higher residence time, reduced linear velocity, and higher tortuosity in the arm of the olfactometer supplied with 4-oxo-(*E*)-2-hexenal showed that this compound modifies the kinetics of some traits of *T. basalis* walking pattern and suggests that it might stimulate the searching behavior of this parasitoid. The parasitoid *T. podisi* was attracted to crude gland extracts of the preferred host (*Euschistus heros*) and also to 4-oxo-(*E*)-2-hexenal. Additionally, this parasitoid responded positively to (*E*)-2-hexenal and to the hydrocarbon tridecane, both of which are defensive compounds released from the metathoracic glands by several stink bugs. The results indicate some degree of specialization in the response of two generalist parasitoid species toward defensive secretions of stink bugs.

Keywords Defensive compounds · Host searching · Host–parasitoid interactions · Kairomones · Host preference · Hymenoptera · Sceolionidae · Hemiptera · Pentatomidae

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Introduction

Parasitoids are known for using semiochemicals as medium- and long-range cues when searching for hosts (Vinson 1985, 1998; Vet and Dicke 1992; Steidle and van Loon 2002; Fatouros et al. 2008). Semiochemicals that originate from the host habitat, the hosts themselves, or indirectly from stages associated to the host can be used by parasitoids during a hierarchical sequence of steps for host location and selection (Vinson 1985).

Egg parasitoids face the challenge of finding hosts that are not, or are barely apparent (eggs). Therefore, they must

rely on semiochemical cues that are more detectable than those from the eggs, such as those from stages of the host that are not suitable for parasitism (adults or immature stages) or from host plants (Vet et al. 1991, 1995; Vet and Dicke 1992; Vinson 1998; Fatouros et al. 2008).

Scelionidae that parasitize eggs from stink bugs can use several types of semiochemicals for long-range localization of habitat, microhabitat, and hosts: volatiles from plants damaged by stink bug oviposition or feeding (Colazza et al. 2004; Moraes et al. 2005a, 2008); sex pheromones (Aldrich 1985, 1995; Borges et al. 1998, 2003; Bruni et al. 2000; Silva et al. 2006); volatile defensive secretions from the metathoracic (adults), or dorsal abdominal (nymphs) glands of stink bugs (Aldrich 1985, 1995; Mattiaci et al. 1993; Borges and Aldrich 1994); or crude whole body extracts of stink bugs (Colazza et al. 1999; Salerno et al. 2006). Volatiles from nonhost stages of stink bugs, such as pheromones or defensive compounds, as well as contact chemicals (traces left by walking insects) also can be used for host location, recognition, and acceptance, thus leading to successful oviposition (Bin et al. 1993; Borges et al. 1999, 2003; Colazza et al. 1999; Conti et al. 2003). Physical stimuli such as visual and resonance cues also may be involved in successful host search (Borges et al. 1999). Laumann et al. (2007) demonstrated that foraging *Telenomus podisi* Ashmead, 1881 can orientate toward hosts by using substrate-borne vibratory signals produced during sexual communication of host stink bugs.

The Scelionidae comprise a large family with about 3,000 described species (Masner 1993) that primarily attack eggs of Heteroptera and Lepidoptera and less frequently are reported as egg parasitoids of Diptera, Orthoptera, Coleoptera, and Araneae (Arias-Penna 2002; Austin et al. 2005). In central Brazil, *Trissolcus basalis* (Wollaston 1958) and *T. podisi* are common parasitoids of stink bug eggs. In soybean agroecosystems in Brazil, *T. basalis* shows high parasitism in eggs of the pentatomid stink bug *Nezara viridula* (L., 1758) and *T. podisi* in eggs of the pentatomid *Euschistus heros* (Fabricius 1794) (Corrêa-Ferreira and Moscardi 1995; Medeiros et al. 1997, 1998; Pacheco and Correa-Ferreira 2000). These field observations were confirmed by Sujii et al. (2002) in a laboratory study, who reported that *T. podisi* showed high parasitism indices in *E. heros* eggs both in no choice and multiple-host choice experiments. In the same set of experiments, *T. basalis* showed high parasitism indices on eggs of *N. viridula* and *Piezodorus guildinii* (Westwood. 1837) in no choice experiments and showed a clear preference for eggs of *N. viridula* in multiple-host choice experiments (Sujii et al. 2002).

Aldrich (1995) pointed out that preferences observed in scelionid wasps may be based on differential long-range attraction to stink bugs allomones. There is evidence that *T. basalis* uses (*E*)-2-decenal, a defensive compound of *N. viridula*, as a kairomone (Mattiaci et al. 1993).

In this study, we tested the following hypotheses by using *T. podisi* and *T. basalis* and five different stink bug species as hosts: (1) Host-searching behavior of the egg parasitoids is differentially influenced by blends from metathoracic glands of the different host stink bug species; (2) Individual components of these blends may be responsible for such a differential response; (3) A differential response to odor from metathoracic glands of hosts is related to the preference for these host species.

We recorded the parasitoids' behavioral responses in a Y-tube olfactometer to crude metathoracic gland extracts of five neotropical stink bugs species and to synthetic standards of individual compounds of the metathoracic glands of different species.

Methods and Materials

Insects Parasitoids used in this work were obtained from a laboratory colony that started from parasitized stink bug egg masses collected near the Embrapa Genetic Resources and Biotechnology Laboratory in Brasilia, DF, Brazil (15°47′ S and 47°55′ W). Insects were maintained in an environmental chamber in plastic cages (25-cm^2-angled-neck tissue culture flasks, ICN Biomedicals) under a 14-h photophase at 26.0± 0.5°C and 65±10% relative humidity. Droplets of pure bee honey were supplied as a food source. Both parasitoid species (*T. basalis* and *T. podisi*) were reared on *E. heros* eggs. Host eggs were exposed to parasitoid females for 24 h and then removed and placed in glass tubes (7.5×1.3 cm) for incubation. Adult parasitoids obtained with these procedures were kept for 24–48 h in the plastic cages described above for mating. Later, the females were separated individually into glass tubes (4×0.5 cm) for use in bioassays. In this way, all females used in the bioassays were similarly experienced.

Gland Extracts To test the influence of the defensive compounds from different host species on the response of the parasitoids, bioassays were performed with the natural blends of metathoracic glands obtained by dissecting adults of different species. Adults of *E. heros*, *N. viridula*, *P. guildinii*, *Chinavia impicticornis* (Stål 1872), and *Edessa meditabunda* (Fabricius 1794) were dissected under a stereoscopic microscope, and the metathoracic glands were extracted with microdissecting forceps. The contents of the dissected glands were extracted in *n*-hexane for 2 h. Then, the tissues were removed, and the volume was adjusted to four glands per milliliter. With this procedure, we obtained metathoracic gland extracts having the defensive compounds in similar concentrations to the range of the solutions of individual compounds used in the bioassays

(next section; M.C.B. Moraes, unpublished data). Before the bioassays, the qualitative compositions of the extracts were checked by gas chromatography (data not shown). Four to five extracts of each species were obtained with this procedure and used in the bioassays.

Chemicals For the bioassays that used individual compounds, solutions of the synthetic standards of (*E*)-2-hexenal, 4-oxo-(*E*)-2-hexenal, tridecane, (*E*)-2-decenal, and undecane were prepared at two concentrations (0.01 and 0.1 mg/ml hexane). These compounds were selected based on the following criteria: (1) compounds commonly found in defensive secretions of neotropical stink bugs and the cosmopolitan, *N. viridula*, i.e., (*E*)-2-hexenal, 4-oxo-(*E*)-2-hexenal, and tridecane (Borges and Aldrich 1992; Aldrich 1995; Zarbin et al. 2000; Moraes et al. 2005b; Borges et al. 2007; Pareja et al. 2007); (2) a compound found as a major component in glands of *N. viridula* and *Chinavia* spp. and as a minor component in *E. heros* and *Thyanta perditor* (Fabricius 1794), i.e., (*E*)-2-decenal (Borges and Aldrich 1992; Moraes et al. 2005b; Pareja et al. 2007); and (3) undecane, a compound found as a major component exclusively in *Edessa meditabunda* (Borges and Aldrich 1992) and *E. rufomarginata* (Howard and Wiemer 1983) and in low amounts (near trace quantities) in *Chinavia ubica* (Rolston 1983), *T. perditor*, *E. heros*, and *P. guildinii* (Borges and Aldrich 1992; Moraes et al. 2005b; Pareja et al. 2007).

All authentic standards were purchased from Sigma-Aldrich (St Louis, MO, USA), Fluka (Buchs., Switzerland), Bedoukian (Danbury, CT, USA), or TCI (Tokyo, Japan). Synthetic 4-oxo-(*E*)-2-hexenal was provided by J.R. Aldrich (USDA, Beltsville, MD, USA).

General Procedures for Olfactometer Bioassays A Y-tube olfactometer constructed with an acrylic block with a Y-shaped cavity (27.5×21.0 cm), placed on top of a translucent glass plate and covered with transparent glass, was used to test the influence of the chemical compounds on the behavior of the parasitoids. The trunk of the apparatus measured 8 cm, plus a 1-cm circular area at the base of the trunk for insect liberation, and each arm measured 7 cm (at an angle of 130°, id 1.5 cm). Charcoal-filtered, humidified air was passed through each arm at 800 ml/min in a push–pull system. The air flow was maintained with two aquarium pumps. The olfactometer was illuminated from above by two fluorescent lamps (40 W) and from below by two infrared lamps (homogenous emission of wavelengths at 950 nm provided by 108 LEDs). The behavior of the insects was recorded by a CCD Sony SPT M324CE camera (fitted with a 4- to 50-mm/F1:1.6 zoom lens with an infrared filter) coupled to the Xbug software (Colazza et al., unpublished). Filter papers (1.5-cm long, 0.5-cm wide) treated with 5 µl of the test solution [gland extract or synthetic compound (25 ml), see above] were introduced into a glass chamber located close to the air entrance in one of the arms. In the other arm, a glass chamber with a filter paper treated with 5 µl *n*-hexane was used as control. To avoid any bias in the parasitoid responses, the arms through which control and treatment odors were presented were inverted every two to three bioassays. The apparatus was cleaned after two to three bioassays with fragrance-free liquid soap, rinsed thoroughly with water, and dried in convector ovens (at 160°C for the glass material and 60°C for the acrylic box).

For each bioassay, a single naïve *T. podisi* or *T. basalis* female (24–48 h adult stage) was introduced at the base of the Y-tube, and its behavior was monitored for 10 min. Insects that had not made a choice after 5 min were considered as nonresponders, and they were not included in the statistical analyses. To test for any bias between the two olfactometer arms, blank tests were carried out, in each set of bioassays, presenting *n*-hexane in both arms (N=30 for each parasitoid species). All bioassays were performed between 1000 to 1600 hours in a room at 26.0±1.0°C. During each bioassay, two parameters were recorded: first choice, measured as the arm of the olfactometer into which the insect entered first for at least 1 cm and remained for at least 20 s; and residence time, measured as the percentage of total bioassay time spent in each arm of the olfactometer.

Bioassays with Defensive Gland Extracts These bioassays were performed to test the response of the parasitoids toward the natural blend of defensive glands of different stink bugs species. Gland extracts that were obtained as described above were used. As a first step, extracts of each species were contrasted with *n*-hexane to test whether the parasitoid showed a significant response to the natural blend of each species. In a second step, the gland extracts of the host species for which a parasitoid showed a positive response were contrasted to investigate in a dual choice test whether the parasitoid showed a preference for one or another host species. For each parasitoid species, 40–50 insects were tested toward each defensive blend extract against *n*-hexane and toward each relevant host species—species pair recording the same parameters as described above.

Bioassays with Synthetic Compounds To test the effect of individual components present in the defensive gland blends, bioassays were performed with authentic standards of the compounds. Compounds tested in bioassays were used in solutions at two concentrations (0.1 and 0.01 mg/ml *n*-hexane). These concentrations were chosen based on previous bioassays described by Mattiaci et al. (1993) and Pires et al. (2001). Each compound was tested using *n*-

hexane as control; 30–55 replicates were performed for each compound at each concentration. The parameters computed during the monitoring were first choice and residence time. Additionally, we investigated the effect of individual compounds on specific parameters of the parasitoids' searching behavior, i.e., linear velocity (mm/s), turning rate (°/cm), and tortuosity. Turning rate is the number of times that the insect changes its route in the olfactometer, and the tortuosity index quantifies insect kinetic movement by the calculation $T=1-mp/tl$, in which mp represents the projection of the track in general straight line of the plain and tl the total length of the track (Borges et al. 2003). The index varies between 0 (zero) for minimal and 1 (one) for maximal tortuosity. Slow linear velocity, high turning rate, and tortuosity may be indicative of searching behavior stimulated by a cue.

Bioassays with Defensive Blends vs. Individual Compounds To test whether individual compounds that elicit positive response in parasitoids have the same effect as complete natural blends from defensive glands, bioassays were performed that contrasted the complete defensive gland blends against individual compounds for which each parasitoid showed a positive response; 40–50 bioassays were performed, and first choice and residence time were recorded.

Statistical Analyses The choices made by the parasitoids in the bioassays were analyzed by logistic regression and estimation of the probability of choosing the test odor. The model fitted contained a factor for the side (left or right) on which the test odor was presented to control for this variability. The hypothesis of no preference (50% first choice to each odor) was tested by means of a χ^2 Wald test. The percentages of the total bioassay time spent in each odor field and in blank tests (residence time) were analyzed by Wilcoxon's matched-pairs test after arcsine transformation of the data. Mean average linear velocity, turning rate, and tortuosity in treatment and control arms for each compound were compared with Student's t test or Mann–Whitney test when the data were not normally distributed. Insects that made a choice but remained immobile for more than 300 s were excluded from the residence time, linear velocity, turning rate, and tortuosity analyses.

Results

Y-tube olfactometer bioassays with metathoracic gland extracts of the five adult stink bug species showed that each parasitoid species responded selectively to the gland extract of its preferred stink bug host species. The egg parasitoid *T. basalis* preferred odor from extracts of *N. viridula* when compared to the control ($\chi^2_1=5.33$, $P=0.021$, $N=46$; Fig. 1a). Residence time in this treatment was higher than in the control ($W=395.0$, $P=0.014$, $N=43$; Fig. 1b). Odors of metathoracic glands of the other stink bug species did not elicit significant preference nor did they affect residence time (Fig. 1a and b). Since *T. basalis* showed a response only to extracts of *N. viridula*, no comparisons were necessary with extracts of other species.

The egg parasitoid *T. podisi* significantly preferred the odor from gland extracts of *E. heros* ($\chi^2_1=13.88$, $P<0.001$, $N=40$; Fig. 2a). When extracts of *N. viridula* were tested,

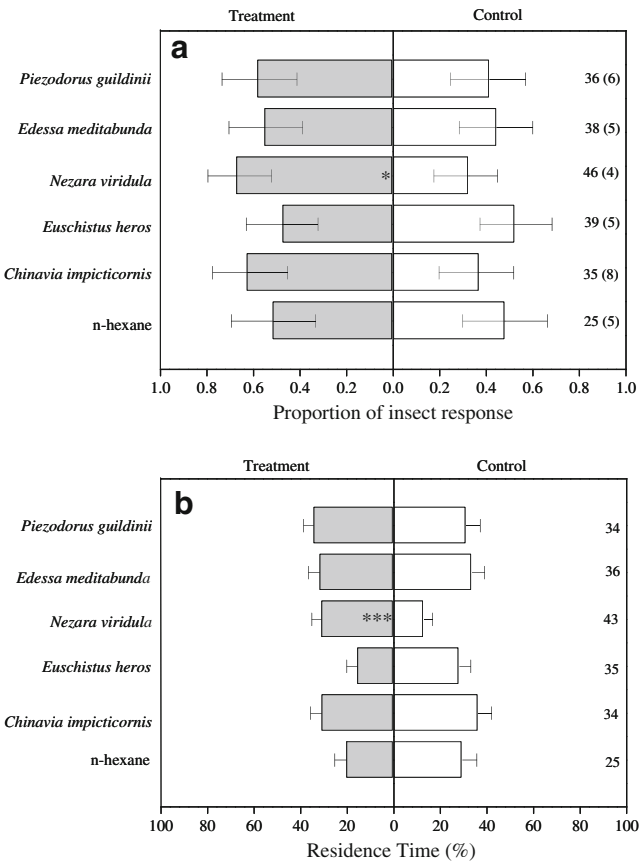

Fig. 1 First choice (**a**) and residence time (**b**) of the parasitoid *T. basalis* in Y-tube olfactometer bioassays with blends of metathoracic glands of different species (four glands per milliliter *n*-hexane) and *n*-hexane as control. Analyses of first choices were carried out by logistic regression and a Wald χ^2 statistic to assess significance. Mean residence time in treatment and control arms was analyzed by Wilcoxon's matched-pairs test. *$0.05>P>0.01$, ***$P<0.001$. *Bars* indicate the mean values of the parameters, and *lines* are the 95% confidence interval for first choice and SE for residence time. *Numbers on the right side of the figures* are the total number of insects tested. *Numbers in brackets in figure* (**a**) represent the number of insects that did not respond to the treatment tested

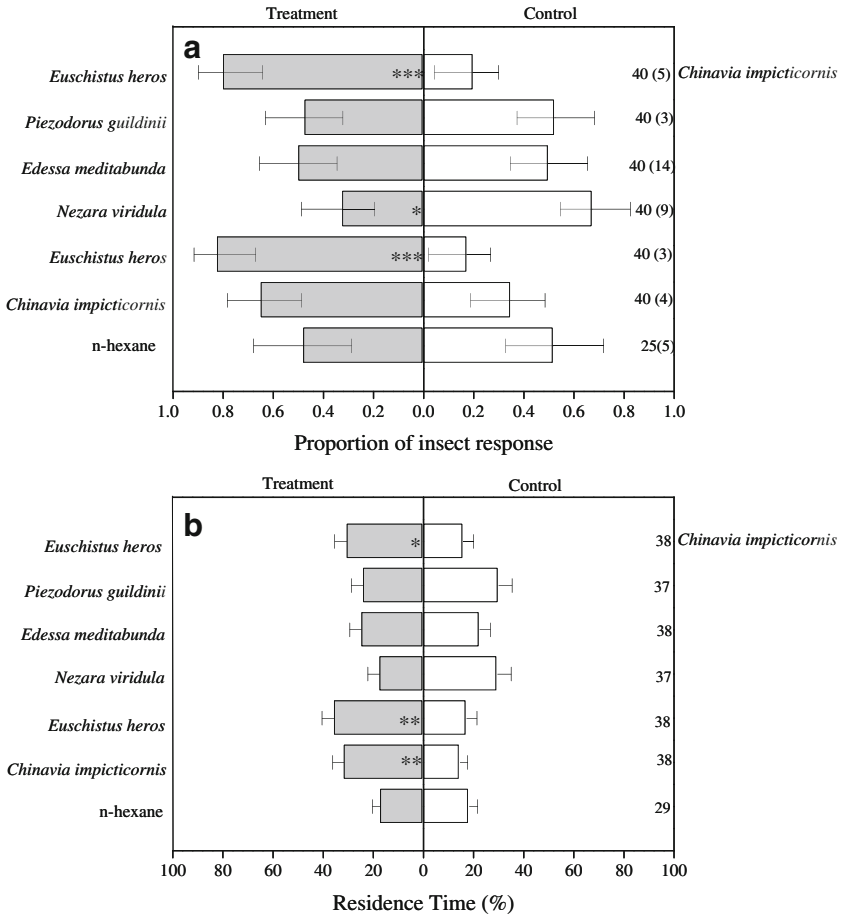

Fig. 2 First choice (**a**) and residence time (**b**) of the parasitoid *T. podisi* in Y-tube olfactometer bioassays with blends of metathoracic glands of different species (four glands per milliliter *n*-hexane) and *n*-hexane as control. Analyses of first choices were carried out by logistic regression and a Wald χ^2 statistic to assess significance. Mean residence time in treatment and control arms was analyzed by Wilcoxon's matched-pairs test. *0.05>*P*>0.01, **0.01>*P*>0.001, and ***$P<0.001$. *Bars* indicate the mean values of the parameters, and *lines* are the 95% confidence intervals for first choice and SE for residence time. *Numbers on the right side of the figures* are the total number of insects tested. *Numbers in brackets in figure* (**a**) represent the number of insects that did not respond to the treatment tested

the parasitoid preferred the control arm of the olfactometer (χ^2_1=4.69, *P*=0.030, *N*=40; Fig. 2a). Parasitoids spent more time in the olfactometer arm supplied with extract of *E. heros* glands than in the control arm (*W*=352.0, *P*=0.011, *N*=38). Odor from extracts of *C. impicticornis* glands (Fig. 2b) also elicited a longer residence time (*W*=349.0, *P*=0.012, *N*=38), whereas the first choice for this odor was not significant (χ^2_1=3.49, *P*=0.062, *N*=40). When the metathoracic gland extracts of *E. heros* were tested against those of *C. impicticornis*, female *T. podisi* showed a clear preference for *E. heros* extracts (χ^2_1=12.30, $P<0.001$, *N*=40 for initial choice and *W*=307.0, *P*=0.026, *N*=38 for residence time; Fig. 2a and b).

The parasitoids showed clear differential responses to the different synthetic compounds tested. *T. basalis* preferred 4-oxo-(*E*)-2-hexenal (χ^2_1=7.68, *P*=0.005, *N*=30) and (*E*)-2-decenal (χ^2=4.53, *P*=0.033, *N*=30) at the higher concentration tested (0.1 mg/ml) against the control (80 and 70% choosing the arm with treatments, respectively; Fig. 3a). This positive response to 4-oxo-(*E*)-2-hexenal was confirmed when the percentage residence time was analyzed. Residence time in the treatment arm supplied with 4-oxo-(*E*)-2-hexenal was higher when compared to the residence time spent in the control arm for the two concentrations tested (*W*=178.0, *P*=0.007, *N*=23 and *W*=271.0, $P<0.001$, *N*=26 for 0.01 and 0.1 mg/ml, respectively; Fig. 3b). (*E*)-2-Hexenal, tridecane, and (*E*)-2-decenal did not show significant effects on *T. basalis* behavior, whereas undecane at the higher concentration tested (0.1 mg/l) caused the parasitoid to spend more time in the control arm (*W*=151.0, *P*=0.015, *N*=22; Fig. 3).

T. podisi showed a positive response to (*E*)-2-hexenal (χ^2_1=4.50, *P*=0.034, *N*=25) and tridecane (χ^2_1=4.61, *P*=0.032, *N*=47) at 0.01 mg/ml and to 4-oxo-(*E*)-2-hexenal at 0.1 mg/ml (χ^2_1=9.22, *N*=0.002, *N*=25). On the other hand, (*E*)-2-decenal and undecane did not elicit a significant response by this species (Fig. 4a). Accordingly, *T. podisi* females showed a higher proportion of residence time in arms of the olfactometer with (*E*)-2-hexenal (0.01 mg/ml) (*W*=211.0, $P<0.001$, *N*=22) and 4-oxo-(*E*)-2-hexenal at the two concentrations (*W*=114.0, *P*=0.03, *N*=20 and *W*=116.0, *P*=0.035, *N*=22 for 0.01 and 0.1 mg/ml, respectively; Fig. 4b). *T. podisi* females did not show higher residence time in the olfactometer arms supplied with

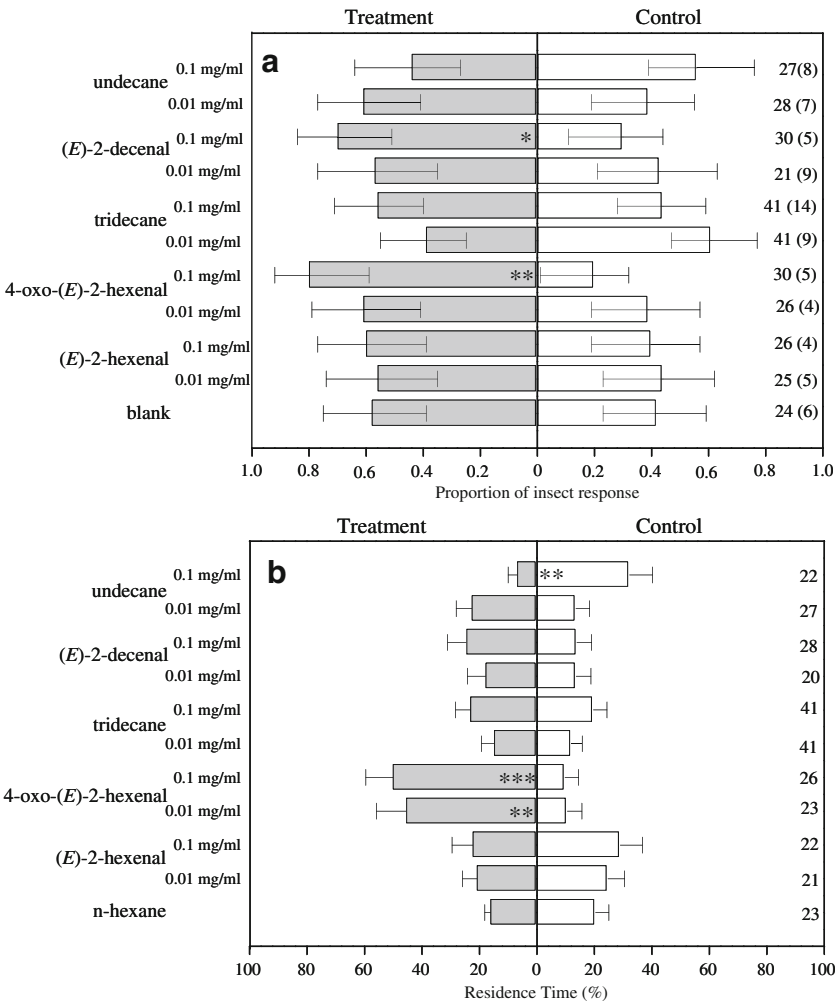

Fig. 3 First choice (**a**) and residence time (**b**) of the parasitoid *T. basalis* in Y-tube olfactometer bioassays with different stink bug defensive compounds as treatments in two dosages (5 µl of 0.01 and 0.1 mg/ml *n*-hexane) and *n*-hexane as control. Analyses of first choices were carried out by logistic regression and a Wald χ^2 statistic to assess significance. Mean residence time in treatment and control arms was analyzed by Wilcoxon's matched-pairs test. *0.05>*P*>0.01, **0.01>*P*>0.001, and *** indicate *P*<0.001. *Bars* indicate the mean values of the parameters, and *lines* are the 95% confidence intervals for first choice and SE for residence time. *Numbers on the right side of the figures* are the total number of insects tested. *Numbers in brackets in figure* (**a**) represent the number of insects that did not respond to the treatment tested

(*E*)-2-decenal, tridecane, or undecane compared to controls (Fig. 4b).

T. basalis showed slower linear velocity (t=2.81, df=22, P=0.01) and a higher tortuosity index (t=2.16 df=23 P=0.04) in olfactometer arms with 4-oxo-(*E*)-2-hexenal (0.1 mg/ml) when compared to hexane (control; Fig. 5). Linear velocity was slower when exposed to tridecane at 0.01 mg/ml compared to the control (Mann–Whitney test U=819.0, N=32, 326, P<0.001), and (*E*)-2-decenal reduced the turning rate of the parasitoid in the control arm (Mann–Whitney test U=318.0, N=15, 17, P=0.008). The other compounds did not show significant effects on *T. basalis* walking patterns parameters (Fig. 5).

T. podisi showed slower linear velocity when exposed to (*E*)-2-hexenal at both 0.1 and 0.01 mg/ml (Mann–Whitney test U=149, N=10, 12, P=0.027, and t test t=2.41, df=20, P=0.021 for 0.01 and 0.1 mg/ml, respectively) and to 4-oxo-(*E*)-2-hexenal at 0.1 mg/ml (Student t test t=2.71, df=20, P=0.013; Fig. 6). Tortuosity was higher when the parasitoid was exposed to (*E*)-2-hexenal (0.01 mg/ml; Student's t test t=2.20, df=24, P=0.037) and 4-oxo-(*E*)-2-hexenal (0.1 mg/ml; Student's t test t=2.97, df=24, P=0.007). Exposure to 4-oxo-(*E*)-2-hexenal led to an increase of the turning rate of *T. podisi* females (Student's t test t=2.14, df=28, P=0.04). Tridecane, (*E*)-2-decenal, and undecane did not show significant effects on *T. podisi* walking pattern parameters (Fig. 6).

When the compounds to which the parasitoids showed significant positive responses were tested against the crude metathoracic gland extract preferred when tested singly, *T. basalis* did not show a preference for the *N. viridula* gland extracts when tested against 4-oxo-(*E*)-2-hexenal at 0.01 mg/ml, but preferred the arm with gland extracts when it was contrasted against 4-oxo-(*E*)-2-hexenal at 0.1 mg/ml (χ^2_1=4.59, P=0.032, N=36). In contrast, when *N. viridula* gland extracts were tested against (*E*)-2-decenal (at 0.1 mg/ml), the parasitoid preferred the arms treated with the aldehyde (χ^2_1=4.59, P=0.032, N=35; Fig. 7 a). In

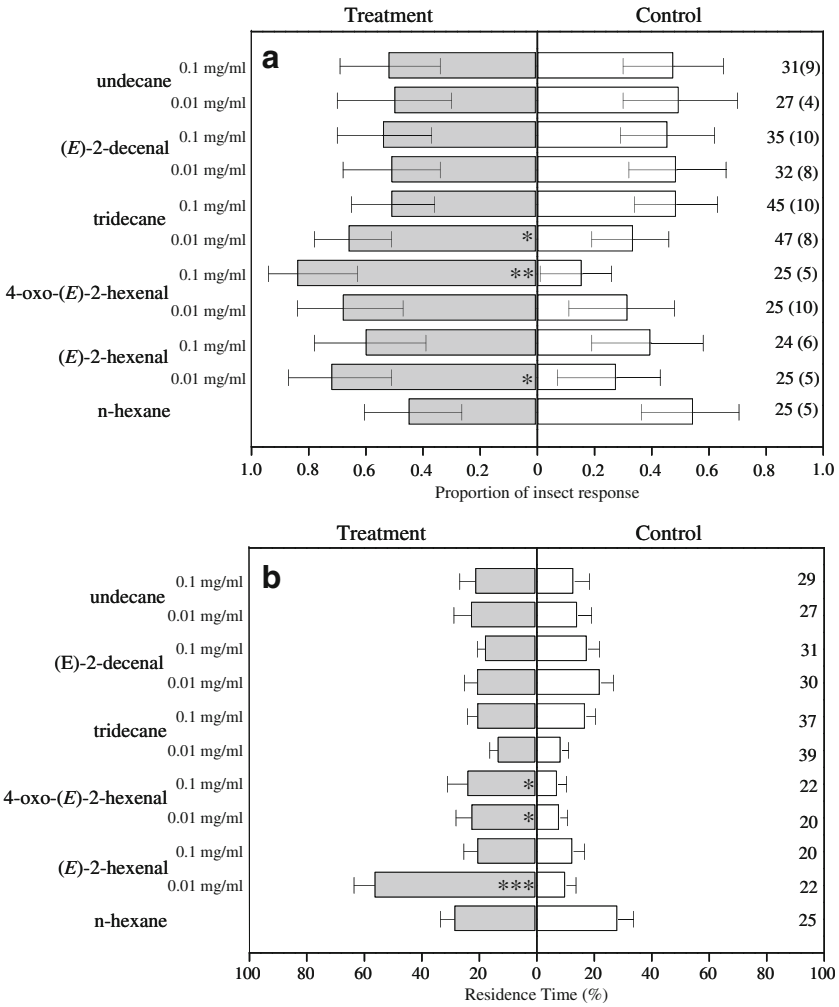

Fig. 4 First choice (**a**) and residence time (**b**) of the parasitoid *T. podisi* in Y-tube olfactometer bioassays with different stink bug defensive compounds as treatments in two dosages (5 µl of 0.01 and 0.1 mg/ml *n*-hexane) and *n*-hexane as control. Analyses of first choices were carried out by logistic regression and a Wald χ^2 statistic to assess significance. Mean residence time in treatment and control arms was analyzed by Wilcoxon's matched-pairs test. *0.05>*P*>0.01, **0.01>*P*>0.001, and *** indicate *P*< 0.001. *Bars* indicate the mean values of the parameters, and *lines* are the 95% confidence intervals for first choice and SE for residence time. *Numbers on the right side of the figures* are the total number of insects tested. *Numbers in brackets in figure* (**a**) represent the number of insects that did not respond to the treatment tested

contrast, the residence time in arms of the olfactometer with the stink bug gland extract or with individual defensive compounds was the same for all combinations tested (Fig. 7b).

The parasitoid *T. podisi* did not prefer odor of *E. heros* glands when this was tested against individual compounds (Fig. 8a, b).

Discussion

Scelionid parasitoids show clear host preferences (Sujii et al. 2002) for stink bug eggs that maximize their biological performance (Pacheco and Corrêa-Ferreira 1998; Kivan and Kilic 2002, 2004; Laumann et al. 2008). Egg parasitoids that search for nonapparent hosts may rely especially on easily detectable cues such as host pheromones or host allomones (Vet and Dicke 1992). Aldrich (1995) postulated that the differential use of adult stink bug host allomones by egg parasitoids should reflect the host preference observed in different species of Scelionidae.

Our results confirm the hypothesis that females of *T. basalis* and *T. podisi* respond differentially to metathoracic gland extracts of different species of stink bugs. Furthermore, the data support the hypothesis that this differential response is related to the preference of hosts reported by Corrêa-Ferreira and Moscardi (1995), Medeiros et al. (1997, 1998), and Pacheco and Correa-Ferreira (2000) from field data, and Sujii et al. (2002) from laboratory experiments. Even when considering that the response of *T. podisi* to *E. heros* gland extracts may be influenced by preimaginal or emergence experience of the parasitoids that were reared on *E. heros* eggs, the response of *T. basalis*, reared on the same host, to gland extracts of *N. viridula* clearly indicates an innate response of this parasitoid to blends of their favorite host. The relevance of individual components of the attractive odor of host metathoracic glands for the parasitoids' host-searching behavior is discussed below.

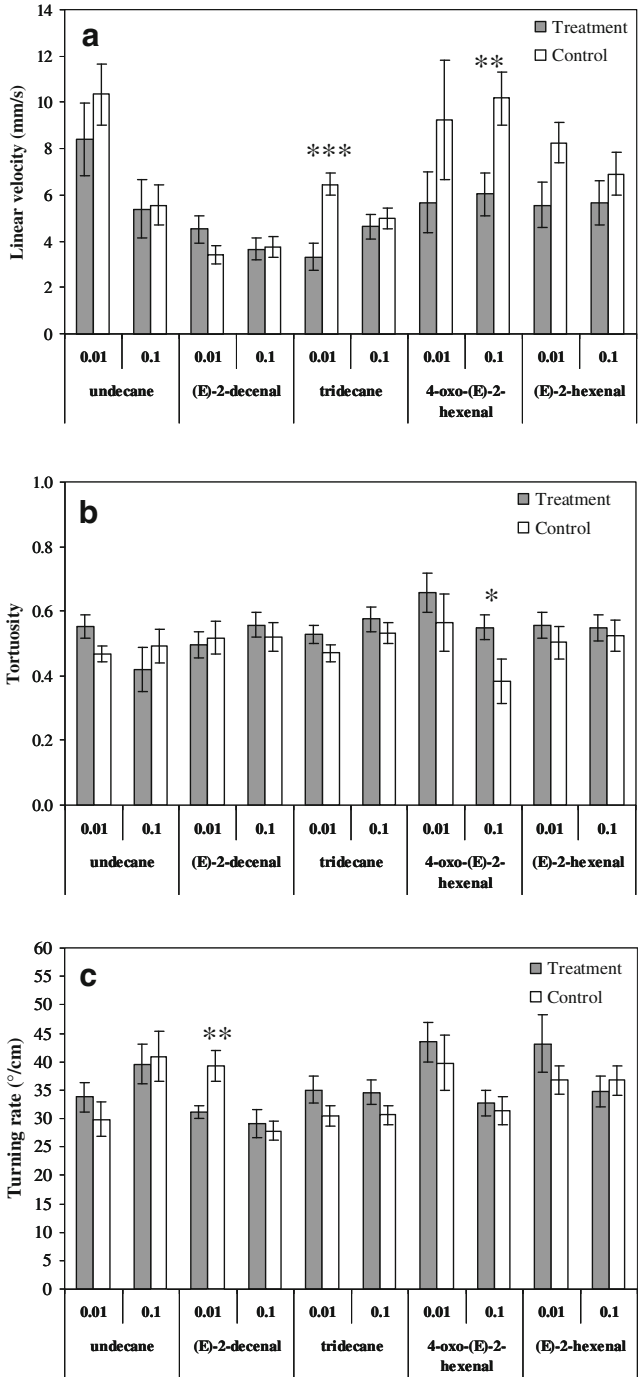

Fig. 5 Walking patterns (mean±SE) of *T. basalis.* **a** Linear velocity, **b** tortuosity, and **c** turning rate. Bioassays were performed in a Y-tube olfactometer with different stink bug defensive compounds as treatments in two dosages (5 µl of 0.01 and 0.1 mg/ml *n*-hexane) and *n*-hexane as control. Means of each treatment and control were compared with Student's *t* test or Mann–Whitney test. *0.05>*P*>0.01, **0.01>*P*>0.001, and ***$P<0.001$

T. basalis showed taxic behavior to two individual compounds tested: 4-oxo-(*E*)-2-hexenal and (*E*)-2-decenal. These findings corroborate the results reported by Mattiaci et al. (1993) who showed that (*E*)-2-decenal is used as a long-range kairomone by *T. basalis*. When testing *T. basalis,* the higher values of residence time, tortuosity, and reduced linear velocity in olfactometer arms supplied with 4-oxo-(*E*)-2-hexenal suggest that this compound can

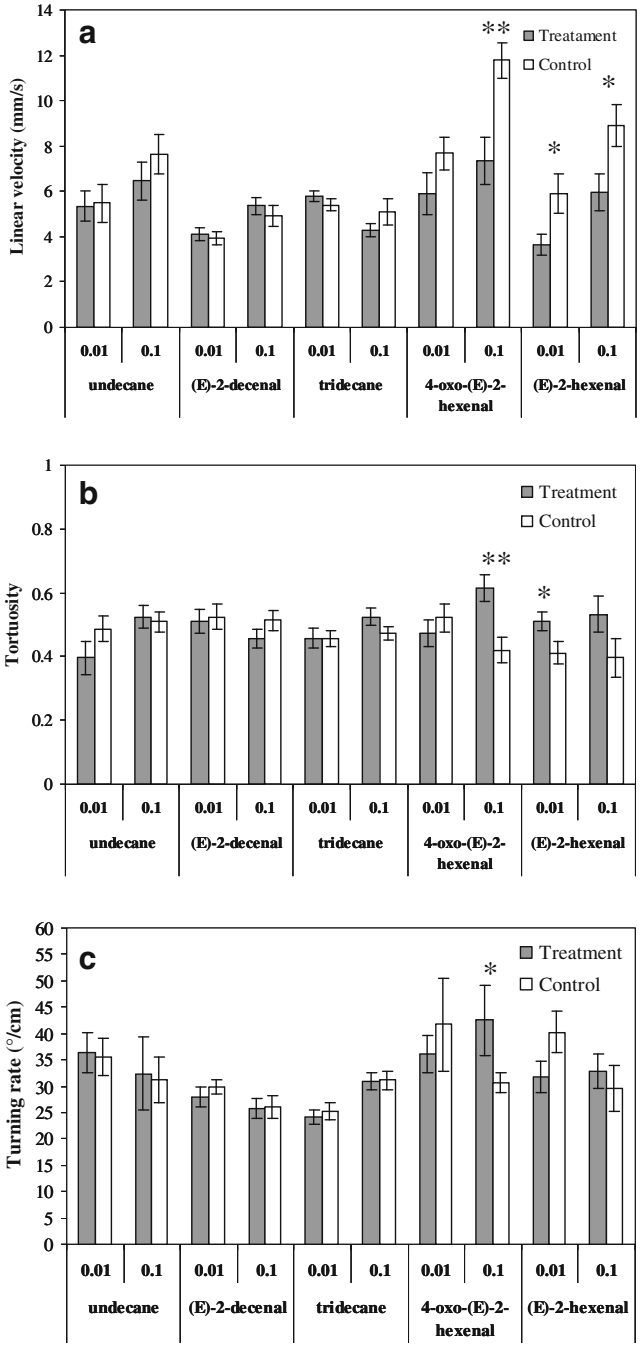

Fig. 6 Walking patterns (mean±SE) of *T. podisi*. **a** Linear velocity, **b** tortuosity, and **c** turning rate. Bioassays were performed in a Y-tube olfactometer with different stink bug defensive compounds as treatments in two dosages (5 µl of 0.01 and 0.1 mg/ml *n*-hexane) and *n*-hexane as control. Means of each treatment and control were compared with Student *t* test or Mann–Whitney test. *0.05>*P*>0.01 and **0.01>*P*>0.001

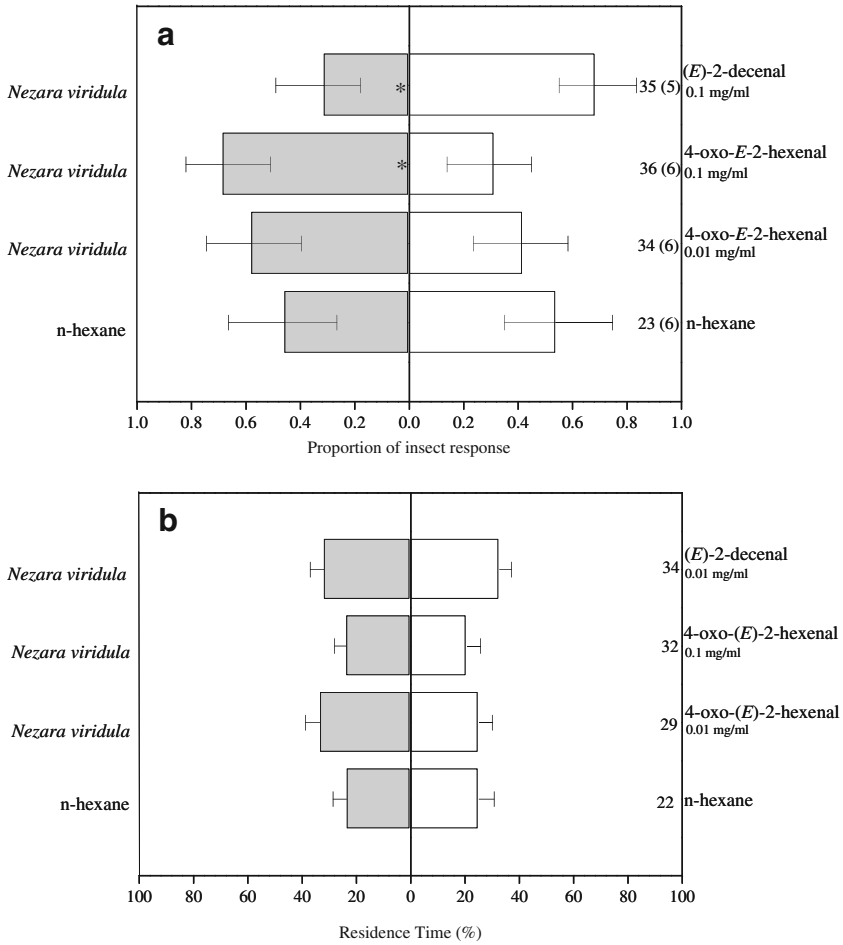

Fig. 7 First choice (**a**) and residence time (**b**) of the parasitoid *T. basalis* in Y-tube olfactometer bioassays contrasting the blends of metathoracic glands of *N. viridula* with different stink bug defensive compounds. Analyses of first choices were carried out by logistic regression and a Wald χ^2 statistic to assess significance. Mean residence time in treatment and control arms was analyzed by Wilcoxon's matched-pairs test. *0.05>*P*> 0.01. *Bars* indicate the mean values of the parameters, and *lines* are the 95% confidence intervals for first choice and SE for residence time. *Numbers on the right side of the figures* are the total number of insects tested. *Numbers in brackets in figure* (**a**) represent the number of insects that did not respond to the treatment tested

stimulate searching behavior of the parasitoid and modify kinetic components of the walking pattern.

T. podisi showed significantly positive response to (*E*)-2-hexenal and 4-oxo-(*E*)-2-hexenal, and these compounds also modified the kinetic search pattern. In contrast, tridecane affected only the initial choice of the parasitoid. The individual responses of *T. podisi* to these compounds are in concordance with those reported by Pires et al. (2001) by using olfactometer bioassays with groups of ten *T. podisi* females.

When both individual compounds and crude blends of metathoracic gland extracts that elicited positive taxic responses by the parasitoids were contrasted, the individual compounds had similar attraction power, suggesting that they can be used by the parasitoid during foraging behavior with the same efficiency as the whole blend released from glands. The only exceptions were 4-oxo-(*E*)-2-hexenal and (*E*)-2-decenal for *T. basalis.* The first compound showed lower attraction than extracts of *N. viridula* glands, and the second was more attractive than the extracts, confirming the strong kairomonal effect previously reported by Mattiaci et al. (1992) for this compound.

Long-range host location and selectivity mediated by semiochemicals has been previously reported for *Trissolcus* spp. responding to volatiles from stink bug nymphs and/or adults in bioassays with living insects (Colazza et al. 1999; Conti et al. 2003, 2004; Salerno et al. 2006; Silva et al. 2006). These previous studies showed that the parasitoids have stronger preference to cues from host stink bug females in a preovipositional state (Colazza et al. 1999). In concordance with the observations here, the parasitoids' response to volatiles from living host insects during host location behavior are related to cues derived from their preferred host or from hosts with old association history (Conti et al. 2004; Salerno et al. 2006).

The response of *T. podisi* and *T. basalis* to 4-oxo-(*E*)-2-hexenal may indicate a general response toward a characteristic compound of Heteroptera (Borges and Aldrich 1992; Aldrich 1995; Pareja et al. 2007). The response to (*E*)-2-decenal (*T. basalis*) and tridecane (*T. podisi*) indicates that even parasitoids with broad host spectra (Orr 1988; Austin et al. 2005) show a certain degree of specialization in the use of cues during searching behavior. The preference of *N. viridula* by *T. basalis* is reflected by its

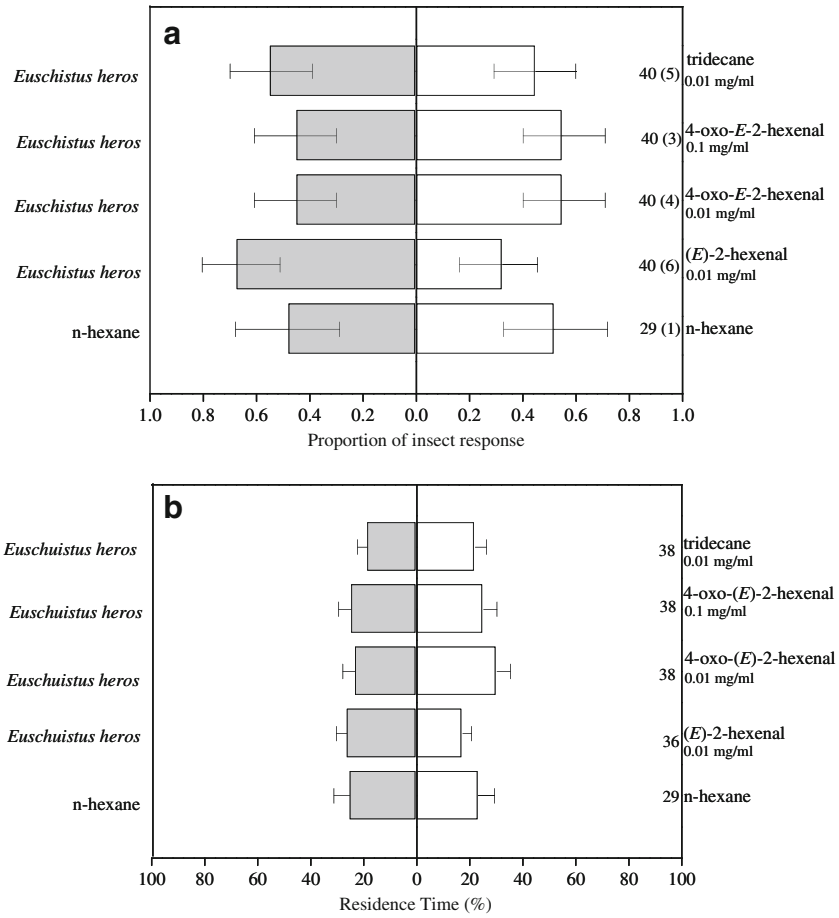

Fig. 8 First choice (**a**) and residence time (**b**) of the parasitoid *T. podisi* in Y-tube olfactometer bioassays contrasting the blends of metathoracic glands of *E. heros* with different stink bug defensive compounds. Analyses of first choices were carried out by logistic regression, and a Wald χ^2 statistic to assess significance. Mean residence time in treatment and control arms was analyzed by Wilcoxon's matched-pairs test. *Bars* indicate the mean values of the parameters, and *lines* are the 95% confidence intervals for first choice and SE for residence time. *Numbers on the right side of the figures* are the total number of insects tested. *Numbers in brackets in figure* (**a**) represent the number of insects that did not respond to the treatment tested

preference to (*E*)-2-decenal, a major component of defensive glands of the host *N. viridula* (Borges and Aldrich 1992). In addition, *Edessa* spp. release undecane as a major compound from their metathoracic glands (Howard and Wiemer 1983; Borges and Aldrich 1992), and they are not known as hosts of *T. basalis* and *T. podisi* in Brazil (Côrrea-Ferreira and Moscardi 1995; Medeiros et al. 1998). Neither parasitoid species showed a significant response toward undecane, suggesting that these parasitoids may use defensive compounds to discriminate unsuitable hosts.

The response of *T. podisi* to (*E*)-2-hexenal may characterize a general response to cues from the habitat where hosts can be found because this compound is usually found in glands of stink bugs: (*E*)-2-hexenal is also a common green leaf volatile present in volatile blends from plants (Hatanaka 1993) and has been reported as a component of soybean headspace (Moraes et al. 2008), which is the major host plant of stink bugs in Central Brazil. This general cue may be replaced, in more advanced steps of host-searching behavior, by specific cues from the host, such as 4-oxo-(*E*)-2-hexenal or tridecane.

A paradigm of host search in parasitic insects predicts that generalist parasitoids use nonspecific cues, while specialists use specific ones (Vet and Dicke 1992; Meiners et al. 2000, Steidle and van Loon 2003). Phoretic scelionids and many other egg parasitoid species with reduced host spectra are known to use species-specific host sex pheromones (Nordlund et al. 1983; Aldrich 1985; Arakaki et al. 1996, 1997; Colazza et al. 1997; Bruni et al. 2000). *T. podisi* uses the sex pheromone of its preferred host, *E. heros* (Sujii et al. 2002; Borges et al. 1998, 1999; Silva et al. 2006). The results presented here indicate that, in addition to this specific response to host sex pheromones, a specific response to host allomones may help the parasitoid find hosts where the performance of their progeny will be high. Thus, even though the scelionid species studied here have quite a broad host spectrum, host species-specific cues might help parasitoids orientate to the most preferred host species.

The use of semiochemicals for behavioral manipulation of parasitoids has been proposed and discussed extensively. In recent years, semiochemicals have also been discussed as a tool to improve biological control (Vet and Dicke 1992; Lewis and Martin 2000; Powell and Pickett 2003). Specific knowledge about host–parasitoid relationships mediated by semiochemicals is important for improving the effectiveness of applications of semiochemicals in integrated pest control.

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Caterpillar Footprints as Host Location Kairomones for *Cotesia marginiventris*: Persistence and Chemical Nature

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Abstract Herbivores walking over the epicuticular wax layer of a plant may leave tracks that disclose their presence to hunting predators or parasitoids. The braconid wasp *Cotesia marginiventris* is a solitary parasitoid of young noctuid caterpillars. It can locate potential hosts from a distance by orienting toward the scent of herbivore-damaged plants. Upon landing on the caterpillars' food plant, the female parasitoid searches for further cues (kairomones) that confirm the presence of a suitable host. In a previous study, we showed that *C. marginiventris* recognizes the chemical footprints of absent *Spodoptera frugiperda* caterpillars on a leaf. Here, we report on the persistence and chemical nature of this host location kairomone. In a series of behavioral assays, we confirmed that caterpillars of *S. frugiperda* leave chemical tracks that elicit characteristic antennation behavior in *C. marginiventris* for up to 2 days. Both hexane extracts of caterpillar footprints and of the larvae's ventral cuticle induced antennation and contained almost identical long-chain hydrocarbons, thus suggesting the prolegs and claspers as the kairomones' main source. A series of linear C_{21} to C_{32} alkanes accounted for ca 90% of all identified compounds. Female wasps showed significant antennation responses on leaves treated with a reconstructed blend of these *n*-alkanes. However, wasp responses were relatively weak. Therefore, we presume that minor compounds, such as monomethyl-branched alkanes, which were also found, may contribute additionally to host recognition.

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Introduction

For parasitoids, semiochemicals play an important role in each stage of locating, recognizing, and accepting a potential host (Vinson 1998; Steidle and Van Loon 2002). Finding a host's microhabitat, e.g., its food plant, is often accomplished by orienting toward herbivore-induced volatile plant compounds (Turlings et al. 1995; Dicke et al. 2003; Heil 2008). Upon landing on a plant, chemical cues of lower volatility that are more closely associated with a host become important. Such chemicals (kairomones) may emanate directly from the host or from host by-products like feces or moth scales (Godfray 1994).

In recent years, it has become clear that small amounts of chemicals remain on the substrate when insects walk over a plant surface. These chemical footprints can be exploited as host location cues by foraging natural enemies, or they may serve other purposes in inter- and intraspecific interactions such as oviposition deterrence or the marking of previously visited flowers in the case of bumblebees (Klomp 1981; Hemptinne et al. 2001; Borges et al. 2003; Conti et al. 2003; Eltz 2006; Colazza et al. 2007; Collatz and Steidle 2008; Rutledge et al. 2008).

The parasitoid *Cotesia marginiventris* Cresson (Hymenoptera: Braconidae) is distributed from North to South America and has been considered for use in augmentative biological control of vegetable pests. As a solitary koinobiont endoparasitoid, it parasitizes early instar lepidopteran larvae belonging to the Noctuidae family (Riddick 2006). Its host location behavior has been investigated intensively, mainly in studies that address the parasitoids' long-range

attraction to host plants by using herbivore-induced volatiles and in the context of how these signals may be influenced by biotic and abiotic factors (Turlings et al. 1995; Cardoza et al. 2002; Gouinguené and Turlings 2002; Rostás et al. 2006; Winter and Rostás 2008). Earlier research also has assessed the parasitoids' responses to contact kairomones. By-products of the host such as feces, larval and pupal cutical material, scales, silk, or oral secretion elicit increased klinokinesis, antennal palpation, or ovipositor probing (Loke and Ashley 1984a, b; Dmoch et al. 1985).

In addition to these cues, Rostás et al. (2008) found that naïve *C. marginiventris* can recognize chemical footprints on the wax surface of leaves produced by walking second-instar caterpillars of *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae). In the current study, we addressed the question of how long such caterpillar footprints can be detected by the female parasitoid, and we investigated their chemistry.

Materials and Methods

Insects Eggs of *S. frugiperda* (Smith) were provided by Bayer CropScience AG (Monheim, Germany) on a weekly basis. After hatching, larvae were reared in plastic boxes (19×9×5.5 cm) and provided with kidney bean-based artificial diet for noctuids (modified from King and Leppla 1984). Insects were kept in a climate chamber with a 15: 9-h L/D photoperiod at 28/25°C (L/D) and 75% humidity. After 5 to 6 days, caterpillars were used either for parasitoid rearing or for experiments. All caterpillars were in their second larval stage in which they are still small and feed gregariously.

A colony of *Cotesia marginiventris* (Cresson) was maintained in the laboratory. For rearing, about 45 *S. frugiperda* larvae were offered to three mated *C. marginiventris* females in a plastic box (20×20×5.5 cm). Wasps were allowed to oviposit for 24 h and were then removed. Herbivore larvae were kept in the boxes until the emergence of the wasp cocoons. Cocoons were removed from the herbivore boxes and transferred to rearing cages (Bugdorm I, Megaview Science Education Services, Taichung, Taiwan). Cages were checked daily for eclosed imagines. Adult parasitoids were provided with water and honey.

Barley (*Hordeum vulgare* cv. Bonus) was cultivated in pots (9-cm diameter). Plants were kept in growth chambers at 300–400 µmol photons m^{-2} s^{-1} light intensity with a day/night cycle of 16:8 h (24:18°C) and 70% relative humidity.

Behavioral Assays A 5.5-cm diameter glass Petri dish covered by a glass plate was used as a test arena for observing the wasps' host location behavior. The Petri dish was surrounded by a 25-cm-high white cardboard cylinder and illuminated by a 25-W light bulb from above. The antennation behavior of single wasps, which had no previous experience with caterpillars, was observed for 10 min using the software Noldus Observer 5.0 (Noldus Information Technologies, Wageningen, Netherlands). Only insects that spent more than 50% of the time in motion were used for analyses. Wasps that showed no antennation behavior throughout the experiment were subjected to a motivation test in which they were provided second-instar host larvae for oviposition. Wasps that laid an egg into at least one caterpillar were included in the analyses as we assumed that they were in oviposition mood even though they did not display antennation behavior. Antennation on the cut edges of the leaves was observed occasionally but not recorded, as *C. marginiventris* is known to respond to green leaf volatiles released by damaged plant tissue (Hoballah and Turlings 2005). Fifteen wasps were observed in each experiment if not stated otherwise. All dual-choice assays were analyzed by using nonparametric Wilcoxon matched pairs test. Wasp responses to hexane and methylene chloride extracts of caterpillars were compared with Bonferroni-corrected Mann–Whitney *U* test.

Persistence of Caterpillar Tracks A clip cage (diameter 20 mm, height 26 mm) covered with gauze on the top and bottom end for ventilation was attached to the upper part of the second leaf of a potted barley plant (*H. vulgare* cv. Bonus). Clip cages contained four caterpillars, which were left to walk over the leaf surface for 15 min. Clip cages were then removed, and the leaf was examined carefully. Only leaves without bite marks, feces, or silk were used in the experiments. Leaves with any kind of residue were discarded. As controls, leaves were used onto which empty clip cages had been attached. The upper part of the leaf (5 cm length) was cut off at different time points (0, 24, 48, and 72 h after clip cage removal). A test and a control leaf section were placed into the arena for observation of parasitoid antennation behavior (see above). Leaf material and wasps were exchanged after each observation.

Chemistry of Caterpillar Tracks In a first bioassay, we tested total body extracts of second-instar *S. frugiperda* assuming that footprint components should be a fragment thereof. Groups of ten caterpillars were shock-frozen in −80°C and then extracted with 1 ml methanol, methylene chloride, and *n*-hexane (Carl Roth GmbH KG, Karlsruhe, Germany), respectively. After 1 min, extracts were filtered (Schleicher and Schüll 595, Dassel, Germany) and evaporated to dryness under a stream of nitrogen. Residues were dissolved in 100 µl of the appropriate solvent and stored at −80°C until used. Pure solvents were used as controls.

Extracts of chemical footprints were prepared by keeping second-instar *S. frugiperda* without food overnight to allow for defecation. Thirty-three to 34 larvae were then left to walk on two microscopic glass slides placed side-by-side, since glass has been shown to provide a good surface for isolating and collecting insect footprints. The slides were covered with a small glass Petri dish (diameter 25 mm, height: 11 mm) to prevent larvae from escaping. All glassware was cleaned thoroughly with distilled water, ethanol, acetone, and *n*-hexane before use. After 1 h, caterpillars were removed, and both glass slides and Petri dish were rinsed with 1 ml *n*-hexane. This procedure was repeated 9×. Three of the extracts were pooled to obtain the footprints of 100 caterpillars. Each of the three pooled extracts was then concentrated to 80 µl under a gentle flow of nitrogen. Glassware without caterpillars was rinsed with *n*-hexane as a control. All extracts were kept at −80°C until used for bioassays or GC-MS analyses.

For comparison of cuticular hydrocarbons, the ventral side of the caterpillar body was extracted with *n*-hexane. Twenty caterpillars were frozen in liquid nitrogen. Then, a 1-µl droplet of *n*-hexane was placed onto a microscopic glass slide with a gas-tight syringe (Hamilton-Bonadaz, Bonaduz, Switzerland). The drop spread over the glass slide covering it as a thin film. By using soft forceps, a caterpillar was placed quickly into the *n*-hexane film before the solvent had evaporated completely. By this procedure, the surfaces of the ventral side including legs, pseudopods, and claspers were extracted. Due to the small size of the second instar, it was not feasible to extract only the extremities.

Observations of wasp antennation responses to the total body and chemical footprint extracts were performed as described above. Single *C. marginiventris* were offered two segments of barley leaves. Each segment was treated with test or control extract (total body 10 µl/leaf, chemical footprints 30 µl/leaf). The solvent was left to evaporate completely before observation started. Wasps were exchanged after each observation. Treated leaves were replaced after five observations (total body) or after each observation (chemical footprints). Fifteen replications were carried out for each treatment.

In another bioassay, wasp antennation responses to the main compounds of the footprint extract were tested. This was accomplished by partially reconstructing the footprint extract with a mixture of standard *n*-alkanes (nC_{21}–nC_{32}, all Sigma-Aldrich, Taufkirchen, Germany). GC analyses of the reconstructed blend were carried out to verify that quantities and ratios of the alkanes were the same as in the original footprint extract that was previously offered to *C. marginiventris*.

Gas Chromatography Hexane extracts were analyzed by gas chromatography and mass spectrometry (Agilent Technologies 6890N Network GC System coupled with a 5973 Network Mass Selective Detector). Three microliters of each sample were injected with an automated injection system in pulsed splitless mode. The column was an Agilent 19091-s933 HP-1 capillary column (length 30 m, diameter 0.25 mm, film thickness 0.25 µm). The oven was held at 150°C for 2 min, and then increased at 5°C min^{-1} to a final temperature of 320°C, which was held for 20 min. Helium (1.5 ml min^{-1}) was used as carrier gas. Linear alkanes were identified by their characteristic EI-MS fragmentation pattern and in addition by using MSD ChemStation (Agilent Technologies) software with the Wiley 275 and NIST 98 mass spectrum libraries. Identities were confirmed further by comparing retention indices and mass spectra with those of authentic *n*-alkane standards (nC_{21}–C_{32}, Sigma-Aldrich, Taufkirchen, Germany). In the case of the monomethyl-alkanes, tentative identification was based on the characteristic fragmentation pattern that indicated the position of the methyl-branch and on the retention indices of the closest *n*-alkanes. Quantities of hydrocarbons were assessed by the external standard method. Calibration curves to determine linearity were obtained from each identified *n*-alkane at four concentrations (1, 5, 10, 25 ng/µl) with three replications per concentration. Linearity was assumed when the regression coefficient R^2 was >0.998. Quantities of monomethyl-alkanes and unidentified compounds were estimated by comparing

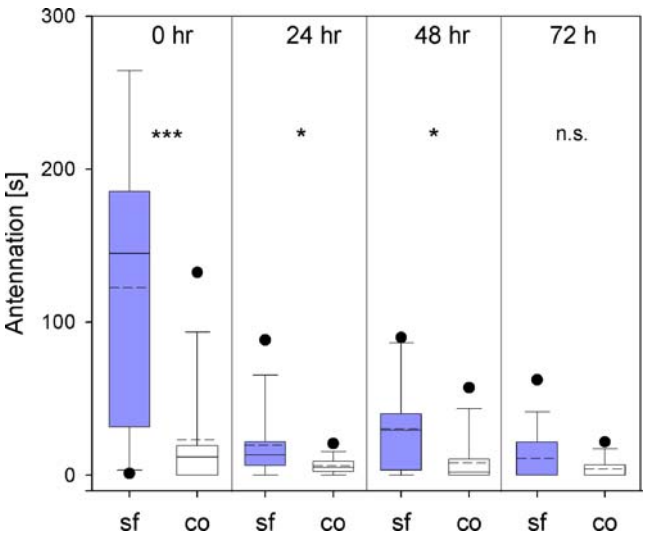

Fig. 1 Antennal drumming of *C. marginiventris* on caterpillar footprints. *Dark boxes* represent barley leaves on which caterpillars of *S. frugiperda* had walked upon for 15 min. *White boxes* represent controls. *Times (0–72 h) above boxes* indicate lag time between removal of caterpillars and observation of wasp behavior. *Boxes* show median (*solid line*) and mean (*dashed line*), 25th and 75th percentiles. *Whiskers* are 5th and 95th percentiles. *Dots* indicate outliers. *Asterisks* denote significant differences: ***$P<0.001$, *$P<0.05$, *n.s.* not significant

peak areas with those of the closest standard *n*-alkane and, thus, were not as absolute as in the case of *n*-alkanes.

Results

Bioassays Assessing Kairomone Persistence The duration of high frequency antennation behavior of *C. marginiventris* observed at various time points after removal of larvae is summarized in Fig. 1. Choice tests showed that female wasps responded most strongly to leaf segments on which caterpillars had been removed right before the experiment started. All parasitoids responded and displayed prolonged antennal drumming on treated leaves (0 h $Z = 3.408$, $P < 0.001$). Significantly longer sequences of antennation on caterpillar-treated leaves were also observed after a time lag of 24 and 48 h, respectively, following the brief exposure to second-instar *S. frugiperda* (24 h $Z = 2{,}981$, $P = 0.003$; 48 h $Z = 2.293$, $P = 0.022$). However, 93% of the observed wasps responded in the 24-h test, while 80% displayed antennal palpation in the 48-h test. After a period of 72 h, wasps still showed a tendency for caterpillar-treated leaf segments, but the difference in antennation time compared to control leaves was no longer significant (72 h $Z = 1.820$, $P = 0.069$). Antennal drumming was observed in 53% of tested wasps.

Chemical Analyses Twenty-one out of 26 detected compounds were identified in the hexane extracts of *S. frugiperda* footprints (Table 1, Fig. 2). All major compounds were odd and even numbered linear alkanes with chain lengths ranging from 21 to 32 carbon atoms (>90% of total). The three most abundant compounds were nC_{25} (13.5%), nC_{26} (10.4%), and nC_{27} (10.3%), representing 34% of the total hydrocarbon extract. Apart from linear alkanes, only monomethyl-branched compounds ($4MeC_{24}$–$4MeC_{28}$ and $3MeC_{25}$–$3MeC_{28}$) could be identified. Comparison with hexane extracts of the ventral side of second-instar cater-

Table 1 Compounds identified from footprint extracts of *Spodoptera frugiperda* caterpillars

ID	Compound[a]	Rt[b]	RI[c]	Amount (ng)[d]		Abundance	Diagnostic ions
				Mean	±SE	(%)	(m/z)
1	*n*-Heneicosane (nC_{21})	14.086	2,100	0.26	0.20	1.1	296
2	*n*-Docosane (nC_{22})	15.779	2200	0.56	0.18	2.6	310
3	*n*-Tricosane (nC_{23})	17.427	2,300	1.34	0.07	5.9	324
4	Unknown	17.965	2,334	0.16	0.10	0.7	287, 302
5	Unknown	18.208	2,349	0.22	0.16	1.0	287, 302, 355
6	*n*-Tetracosane (nC_{24})	19.024	2,400	2.11	0.10	9.2	338
7	4-Methyl tetracosane ($4MeC_{24}$)	20.007	2,464	0.06	0.01	0.3	71, 309
8	*n*-Pentacosane (nC_{25})	20.566	2,500	3.08	0.27	13.5	352
9	4-Methyl pentacosane ($4MeC_{25}$)	21.518	2,564	0.17	0.10	0.7	71, 323
10	3-Methyl pentacosane ($3MeC_{25}$)	21.654	2,573	0.05	0.01	0.2	57, 337
11	*n*-Hexacosane (nC_{26})	22.054	2,600	2.39	0.04	10.4	366
12	4-Methyl hexacosane ($4MeC_{26}$)	22.970	2,664	0.20	0.06	0.9	71, 337
13	3-Methyl hexacosane ($3MeC_{26}$)	23.107	2,673	0.10	0.02	0.4	57, 351
14	*n*-Heptacosane (nC_{27})	23.488	2,700	2.36	0.51	10.3	380
15	4-Methyl heptacosane ($4MeC_{27}$)	24.375	2,764	0.06	0.08	0.2	71, 351
16	3-Methyl heptacosane ($3MeC_{27}$)	24.521	2,774	0.11	0.06	0.5	57, 365
17	*n*-Octacosane (nC_{28})	24.877	2,800	1.54	0.28	6.7	394
18	4-Methyl octacosane ($4MeC_{28}$)	25.737	2,864	0.06	0.02	0.3	71, 365
19	3-Methyl octacosane ($3MeC_{28}$)	25.868	2,874	0.02	0.03	0.1	57, 379
20	*n*-Nonacosane (nC_{29})	26.217	2,900	1.56	0.63	6.8	408
21	Unknown	26.942	2,956	0.13	0.10	0.6	391, 406, 430
22	Unknown	27.039	2,963	0.03	0.03	0.1	281, 381
23	*n*-Triacontane (*n*C30)	27.516	3,000	0.61	0.09	2.7	422, 423
24	*n*-Hentriacontane (*n*C31)	28.776	3,100	0.41	0.08	1.8	436
25	Unknown	29.556	3,164	0.05	0.07	0.2	387, 402
26	*n*-Dotriacontane (*n*C32)	29.998	3,200	0.16	0.01	0.6	450

[a] *n*-alkanes were identified by comparison of retention times and mass spectra with authentic standards. Methylbranched alkanes were tentatively identified by diagnostic ions resulting from favored fragmentation at branching points
[b] *Rt* retention time
[c] *RI* retention index
[d] Amount = Footprint compounds (ng) are equivalent to four caterpillars [L_2]

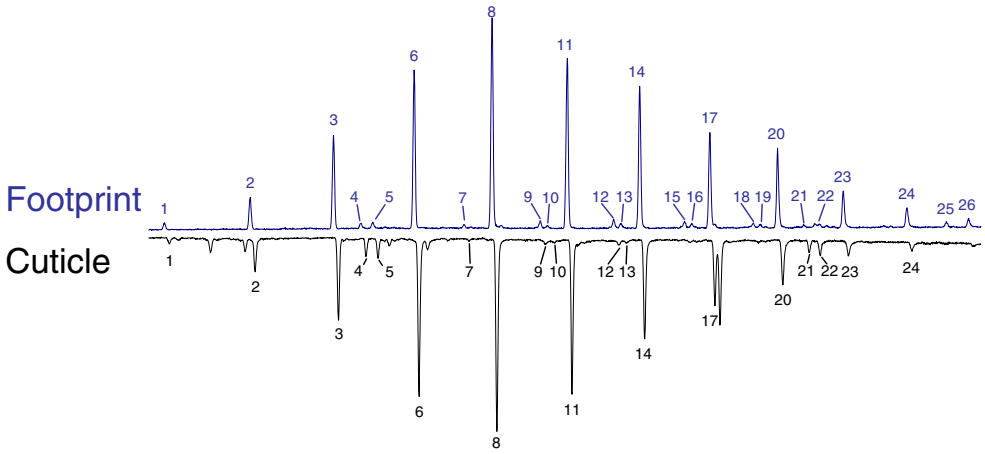

Fig. 2 Chromatograms of footprint and cuticle extracts. Compounds are listed by their numbers in Table 1

pillars showed that the footprint compounds were present in the same ratios in the body extract, as well.

Bioassays that Assessed Kairomone Chemistry Female *C. marginiventris* responded equally well to hexane and methylene chloride extracts of whole caterpillars (hexane vs. control, $Z=2.52$, $P=0.012$, methylene chloride, $Z=2.52$, $P=0.012$; hexane vs. methylene chloride, Mann–Whitney U test, $U=112.5$, $P=0.999$; Response: 53% of wasps in both treatments). Virtually, no response was observed when leaves were treated with methanol extracts of the herbivore. One parasitoid showed brief antennal drumming on the test leaf, another individual responded to the control.

In a bioassay that tested hexane extracts of caterpillar footprints, 67% of the observed wasps elicited characteristic antennation behavior (Fig. 3a). The footprint extract was more attractive than the control extract ($Z=2.803$, $P=0.005$). The mix of *n*-alkanes (C_{21-32}) applied to the barley surface elicited a weaker but still significant response in *C. marginiventris* ($Z=1.991$, $P=0.046$). Forty percent of the tested individuals responded in the experiment (Fig. 3b).

Discussion

The parasitoid *C. marginiventris* responds to chemical residues (footprints) of its lepidopteran host *S. frugiperda* with characteristic host recognition behavior, displayed as antennal drumming on the substrate (Rostás et al. 2008). However, the persistence and chemistry of these footprints had not yet been investigated.

Our experiments showed that the host location kairomone was persistent for a relevant period of time. Only 15 min of walking by four small caterpillars left sufficient amounts of infochemicals on the plant surface to induce a clear antennation response 48 h after caterpillars had been removed. Following a lag time of 72 h, antennation time on the treated area was no longer significant. These results suggest that host-derived chemicals are uncharged lipophilic compounds of low volatility, as only such chemicals can be sorbed onto the leaf surface and stay there for some time due to the lipophilic properties of the plant cuticle (Müller and Riederer 2005). Little is known about the persistence of insect walking tracks. Hemptinne et al. (2001) reported that females of the ladybird *Adalia bipunctata* avoid laying eggs on filter paper contaminated with conspecific larval tracks for at least 10 days. Tracks produced by ladybird larvae were predominantly straight-chain and methyl-branched alkanes in the C_{21} to C_{33} range. The bioactivity of footprints is expected to vary with the nature and concentration of deposited chemicals, the substrate, and the perceiving insect species. Most insect tracks reported, so far, consist of long-chain alkanes and alkenes (Kosaki and Yamaoka 1996; Hemptinne et al. 2001; Votsch et al. 2002; Nakashima et al. 2004; Eltz 2006; Colazza et al. 2007). Thus, their persistence can be expected to be in the range of days rather than hours. An exception, however, was found in the tracks of the grain beetle *Oryzaephilus surinamensis* that can be detected by the parasitoid *Cephalonomia tarsalis* for only 30 min (Collatz and Steidle 2008). Aldehydes rapidly oxidizing to fatty acids were the active components in the footprints of the beetle (Collatz, personal communication).

Extracts with methylene chloride and hexane, but not methanol, of entire *S. frugiperda* caterpillars elicited clear antennal drumming responses in female *C. marginiventris* to equal extents. This shows that wasps use apolar cuticular components for host recognition and suggests that caterpillar footprints should be lipophilic, as well. Further bioassays with hexane extracts of *S. frugiperda* footprints have confirmed this notion, as wasps responded positively to the offered residue chemicals. The major compounds that account for more than 90% of the footprint extract are a homologous series of saturated alkanes ranging from heneicosane (nC_{21}) to dotriacontane (nC_{32}) with pentacosane (nC_{25}) being the most abundant. Monomethyl-branched alkanes and five unidentified compounds also were present,

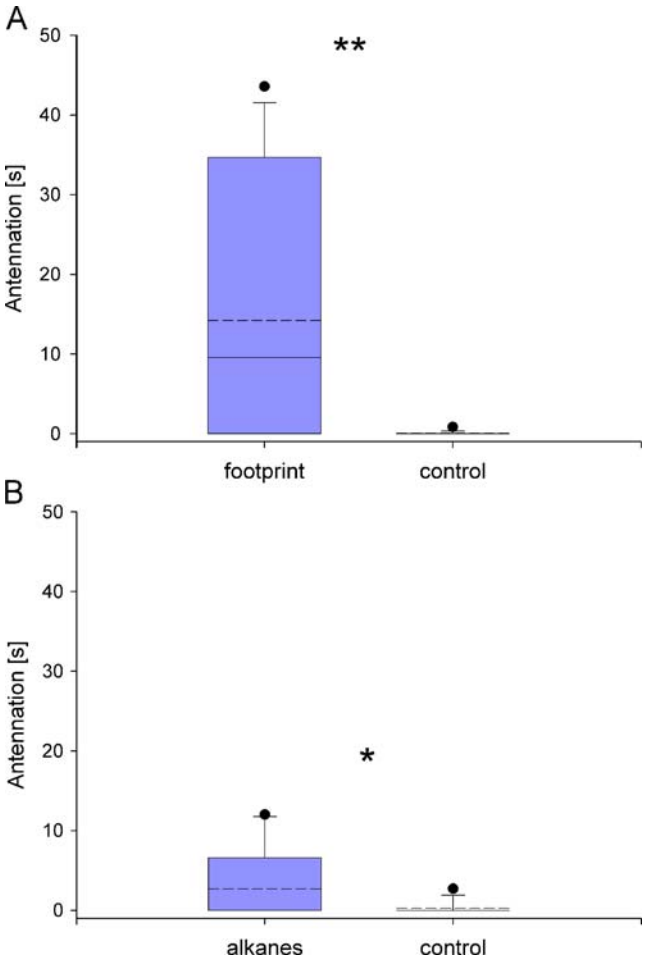

Fig. 3 Antennal drumming of *Cotesia marginiventris* on **a** hexane extracts of caterpillar footprints and **b** *n*-alkanes found in footprint extract. *Dark box* represents leaves treated with footprint extract or *n*-alkanes, respectively. *White box* represents hexane control. *Boxes* show median (*solid line*) and mean (*dashed line*), 25th and 75th percentiles. Whiskers are 5th and 95th percentiles. *Dots* indicate outliers. *Asterisks* denote significant differences: $*P<0.05$, $**P<0.01$

but occurred only as minor compounds. An almost identical pattern of chemicals was found in the hexane extract of the caterpillar's ventral cuticle. Thus, we suggest that footprint compounds may be of cuticular origin. Thoracical legs, prolegs, and claspers were observed to be in contact with the substrate (Fig. 4), and it seems that the most likely source for the kairomone should be the caterpillar's prolegs and claspers. These extremities are covered by a cuticle and represent a larger surface in contact with the leaf than the claws of thoracical legs.

With regard to compositions and ratios of cuticular and footprint alkanes, remarkably similar findings have been reported from the pentatomid bug *Nezara viridula* (Colazza et al. 2007). *Trissolcus basalis*, a parasitoid of this bug, uses the deposited hydrocarbons to locate host eggs and may even distinguish between male and female residues by the presence or absence of *n*-nonadecane. In two other species of the genus *Cotesia* (*C. kariyai* and *C. plutellae*), linear, monomethyl- and dimethyl-branched alkanes (C_{20}–C_{40}) from the cuticula of lepidopteran hosts also are known to serve as host recognition cues upon contact with the host (Ohara et al. 1996; Roux et al. 2007). Whether some of these compounds are deposited on the substrate, where they can be exploited as host location kairomones, remains to be investigated.

In a final experiment, we tested the wasps' response to the main compounds of the footprint extract. Commercially available $n\mathrm{C}_{21}$–$n\mathrm{C}_{32}$ alkanes were used to partially reconstruct the chemical footprint extract in the same amounts and ratios as had been tested in the preceding bioassay. *C. marginiventris* females showed significant antennal drumming on leaves treated with this *n*-alkane blend. However, the response was considerably weaker, suggesting that some minor components, such as the methyl-branched alkanes or other unidentified chemicals are important for host recognition, as well. Our observation is consistent with studies on *N. viridula* and its egg parasitoid *T. basalis* where a reconstructed blend of straight-chain hydrocarbons extracted from the host also induced only weak arrestment responses in *T. basalis* females (Colazza et al. 2007). Methyl-branched alkanes and alkenes often occur in insect hydrocarbons, and sometimes, they have behavioral activity as contact pheromones (Ginzel et al. 2003). In plant waxes, however, they can be detected only in trace amounts, if at all. This and the fact that *n*-alkanes are common plant wax compounds suggests that minor constituents may enable wasps to detect insect hydrocarbons against a background of similar plant hydrocarbons. In the experiments described here, barley leaves were used as a surface for caterpillars to walk on. The epicuticular wax of this plant contains very small proportions of *n*-alkanes with heptacosane ($n\mathrm{C}_{27}$) as the only overlapping compounds that are also present in the chemical footprint extract (Rostás et al. 2008). At least in this case, it can be assumed that linear alkanes derived from the caterpillars can be recognized as host-specific kairomones. Future research will need to assess whether the degree of overlap between plant and insect hydrocarbons plays a role in the perception of chemical footprints on

Fig. 4 *Spodoptera frugiperda* walking on a barley leaf. Note that only thoracical legs, prolegs, and claspers touch the substrate

different waxy surfaces. Depending on the specific wax composition, the detection of chemical footprints on different plant surfaces may be easier or more difficult for hunting parasitoids and predators.

In summary, we confirmed that parasitoids use footprint chemicals deposited on the epicuticular wax layer of plants as low-volatile cues to find their host in its microhabitat. Tracks produced by caterpillars consist mostly of linear alkanes and minor amounts of branched alkanes that presumably originate from the cuticle of prolegs and claspers. On barley leaves, these footprints can be detected by *C. marginiventris* for a maximum of 2 days. Our findings add to the growing body of literature demonstrating the importance of insect-derived chemicals deposited on plants. These serve as a bridge in time and thus mediate numerous interactions. We expect that future research will establish the significance of residual chemicals in regulating behavior—an aspect of insect ecology that awaits full appreciation.

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Defensive Role of Tomato Polyphenol Oxidases against Cotton Bollworm (*Helicoverpa armigera*) and Beet Armyworm (*Spodoptera exigua*)

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Abstract Tomato (*Solanum lycopersicum*) polyphenol oxidases (PPOs), enzymes that oxidize phenolics to quinones, have been implicated in plant resistance to insects. The role of PPO in resistance to cotton bollworm [*Helicoverpa armigera* (Hübner)] and beet armyworm [*Spodoptera exigua* (Hübner)] (Lepidoptera: Noctuidae) was evaluated. Consumption, weight gains, and mortality of larvae feeding on foliage of transgenic tomato lines overexpressing PPO (OP lines) and of larvae feeding on foliage of transgenic tomato lines with suppressed PPO (SP lines) were compared with consumption, weight gains, and mortality of larvae feeding on non-transformed (NT) plants. Increases in foliage consumption and weight gains were observed for cotton bollworms feeding on leaves of SP plants compared to NT and OP plants. PPO activity was negatively correlated with both weight gains and foliar consumption of cotton bollworm, substantiating the defensive role of PPO against this insect. Similarly, beet armyworm consumed less foliage (both young and old leaves) from OP plants than SP plants. Larvae feeding on OP leaves generally exhibited lower weight gains than those feeding on SP leaves. These results indicate that tomato PPO plays a role in resistance to both cotton bollworm and beet armyworm.

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Introduction

Beet armyworm [*Spodoptera exigua* (Hűbner)] and cotton bollworm [*Helicoverpa armigera* (Hűbner)] are two of the most destructive and widely distributed Lepidopteran pests of many important crops, including tomato, sugar beet, legumes, cotton, maize, soybean, tobacco, pepper, alfalfa, potato, onion, sunflower, and citrus, as well as many weeds (Center for Integrated Pest Management 1982). Control measures for beet armyworm and cotton bollworm are both expensive and unsuccessful because both pests are fecund and polyphagous and because both have developed resistance against insecticides. The most successful crop protection recommendations include monitoring of pest populations, implementation of integrated pest management with conservation and augmentation of parasites and predators, the use of sex pheromone traps and hormone inhibitors, and the use of transgenic Bt crops (Haffman et al. 1996; Summers et al. 1996, 2004; Sikora 2000; Godfrey and Kaffka 2003; Manjunath 2004; Mellet and Schoeman 2004; Tillman and Bell 2006). However, a recent study confirmed the development of seven to 50-fold resistance to Bt toxins (Cry1Ac) from *Bacillus thuringiensis* in cotton bollworm within 17 to 21 generations (Akhurst et al. 2006). Therefore, development of other effective control methods must be further investigated for future use.

Polyphenol oxidases (PPOs) are copper metalloenzymes that catalyze two distinct reactions: the *o*-hydroxylation of monophenols to *o*-diphenols (EC 1.14.18.1) and the

oxidation of *o*-dihydroxyphenols to *o*-diquinones (EC 1.10.3.2; Steffens et al. 1994). Quinones are highly reactive intermediates that rapidly polymerize, leading to the formation of black or brown pigments that affect the quality and nutritional value of many fresh and processed products (Mayer and Harel 1991; Friedman 1997). In most plant species, PPO and its mono and/or *o*-diphenolic substrates are stored separately (PPO in plastids, phenolics in the vacuole) and come into contact only after cellular decompartmentalization occurs as a consequence of wounding, senescence, or attack by insects or pathogens. Under acidic conditions (pH<4), quinones undergo a reversed disproportionation reaction to form semiquinone radicals, leading to the generation of reactive oxygen species (ROS). At higher pHs, black or brown pigments are produced from nucleophilic Michael addition reactions of quinones, and the covalent 1,4-addition of quinones to cellular nucleophiles may also occur (Guyot et al. 1995, 1996). These secondary reactions may lead to damage of DNA, protein, and/or lipid (Steffens et al. 1994).

PPOs, which are ubiquitous in angiosperms, may have evolved to the point of now fulfilling diverse functions in different plant species. Several physiological roles for PPO have been suggested that include biosynthesis of pigments (aurones or betalains) and 8 to 8′ lignans, production of phenolic signaling molecules, regulation of plastidic oxygen levels, and plant defense (Mayer and Harel 1979; Vaughn et al. 1988; Goldman et al. 1998; Nakayama et al. 2000; Li and Steffens 2002; Cho et al. 2003; Thipyapong et al. 2004a; Wang and Constabel 2004; Gandía-Herrero et al. 2005). In tomato, the PPO gene family is clustered on chromosome eight and consists of seven members including PPO A, A′, B, C, D, E, and F (Newman et al. 1993). PPOs are accumulated in leaves, roots, stems, and flowers of tomato with particularly high levels in young tissues that are vulnerable to diseases and insect pests. These PPO gene members are expressed differentially in various vegetative and reproductive tissues and in response to abiotic and biotic stresses (Thipyapong et al. 1997, 2004a, b; Thipyapong and Steffens 1997). The constitutive and induced expression patterns of PPOs suggest the possible involvement of PPOs in plant defense. In particular, a variety of evidence has implicated PPO in defense against diverse diseases and insect pests. PPO activity levels are associated with disease resistance or are induced in response to pathogen infection in several plant–pathogen interactions (Tyagi et al. 2000; Banga et al. 2004; Campos et al. 2004; Krishnamoorthy et al. 2004; Raj et al. 2006). In addition, a role for PPO in resistance to *Pseudomonas syringae* pv. *tomato* has been demonstrated unequivocally using transgenic tomato plants with modified PPO expression; overexpression of PPO led to increased resistance, while PPO suppression led to hypersusceptibility (Li and Steffens 2002; Thipyapong et al. 2004a). Recently, these transgenic tomato plants also were used as a platform to establish the defensive role of PPO against common cutworm [*Spodoptera litura* (F.)] (Thipyapong et al. 2006; Mahanil et al. 2008). We report the effect of PPO on foliar consumption, larval weight gains, and mortality of two additional Lepidopteran insects, cotton bollworm and beet armyworm, using genetically modified tomato plants with altered PPO expression in an otherwise identical genetic background.

Methods and Materials

Plant Material and Growth Conditions Five tomato (*Solanum lycopersicum* cv. Money Maker) genotypes varying in PPO activity levels were used for this study: a non-transformed (NT) line, two independent antisense PPO transgenic lines, A14-6 and A19-3, with suppressed PPO activity (SP lines), and two independent sense PPO transgenic lines, S-18 and S-28, overexpressing PPO activity (OP lines). These SP and OP transgenic plants have been described in detail previously (Li and Steffens 2002; Thipyapong et al. 2004a) and were from the F_8–F_9 generations. Briefly, transformation constructs used for generating these SP and OP tomatoes were made by ligating a 2-kb potato PPO complementary DNA (Hunt et al. 1993), exhibiting 68–91% DNA sequence similarity to the seven tomato PPO genes (Newman et al. 1993), into transformation vector pBI 121 (Clontech) in the antisense orientation relative to the CaMV 35S promoter or by ligating the same gene in the sense orientation relative to the CaMV 35S promoter within cloning vector pART 7 (Gleave 1992). The constructs were introduced into *Agrobacterium tumefaciens* that was then used to transform cotyledons and hypocotyls of tomato. The SP lines A14-6 and A19-3 have approximately two to 40-fold reduced PPO activity and lack or have low levels of immunologically detectable PPO in leaf homogenates. Neither endogenous nor antisense PPO messenger RNA is detectable in leaves or flowers of A14-6 (Thipyapong et al. 2004a). The OP lines S-18 and S-28 contain two to tenfold increased PPO activity and immunodetectable PPO and show up to 30-fold increases in PPO transcripts (Li and Steffens 2002). No effect on plant growth and development was observed from PPO modification. The transgenic plants exhibited morphology and plant vigor similar to NT plants and flowered and set seeds normally (Steffens et al. 1994; Li and Steffens 2002; Thipyapong et al. 2004a, b; Mahanil et al. 2008). In addition, the total foliar protein contents of these transgenic plants were not significantly different from those of NT plants (Mahanil et al. 2008). All tomato genotypes were grown in 1:1 peat moss/soil mix in a laboratory culture room with a 13-h photoperiod at a

photosynthetic photon flux density of 200–300 μmol m^{-2} s^{-1} at plant height and 26–30°C day/night temperature.

Cotton Bollworm [Helicoverpa armigera (Hübner)] Feeding Assays Cotton bollworm larvae were collected from the Suranaree University of Technology Farm and from cotton fields in Lop Buri province, Thailand and reared on artificial diet until pupation. The performance of cotton bollworm larvae when feeding on detached leaves of tomato genotypes varying in PPO activity levels was evaluated. Leaf position was counted from the top of the plants with the topmost (youngest) leaf node as node 1. Male and female adults were randomly mated, and the resulting eggs were allowed to eclose. Neonates were reared on artificial diet for 4 days. The 4-day-old larvae were then weighed and divided equally onto detached leaves taken from nodes 4 and 8 of four tomato genotypes, NT, A14-6, A19-3, and S-18. Each larva was placed onto detached leaves in an individual plastic container (6-cm diameter) and allowed to feed for 7 days. The initial areas of leaves were measured with a leaf area meter. The petioles of leaves were wrapped in wet cotton, and leaf material was changed every 2 days, or sooner if they were almost completely eaten, so that larvae were never food limited. After 7 days of feeding, weight gains of larvae (assuming that larvae in all treatments contained similar amounts of food in their guts at the end of the experiment), larval mortality, and remaining leaf areas were recorded.

Five replications were used for each treatment (genotype). Each replication consisted of three larvae, each in its own plastic container, fed leaves from five to ten plants grown near each other in the plant culture room for the entire experiment. Thus, a total of 15 larvae were used per treatment per experiment. For statistical analysis, mean weights and leaf areas consumed were calculated for the three larvae in each replication, and these mean weights and areas were used in the analyses. Leaf areas were measured when leaves were changed, and after the 7 days of feeding, the leaf areas consumed were calculated from the original leaf areas minus the leaf areas remaining after feeding. Percent mortality was calculated by the following formula: Percent mortality = (number of dead larvae/number of total larvae) × 100. The effects of plant genotype and leaf age (node) on larval weight gains and leaf areas consumed were evaluated using analysis of covariance (ANCOVA) with initial larval weight as the covariate (Raubenheimer and Simpson 1992; Horton and Redak 1993). Relative growth rates (RGRs) were calculated by dividing weight gains by the product of mean larval weight and feeding duration in days, as appropriate for assays in which larvae molted one or more times (Farrar et al. 1989). A two-way analysis of variance (ANOVA) of larval RGRs was conducted. All analyses were conducted using PROC MIXED in SAS (SAS Institute 2003).

In addition, the relative importance of pre-ingestive effects (e.g., feeding deterrence) vs. post-ingestive effects (e.g., antibiosis) of PPO on larval weight gains was investigated by ANCOVA using weight gain as the response variable and leaf consumption as the covariate. This analysis was conducted using PROC MIXED in SAS (Horton and Redak 1993).

In a separate experiment to further evaluate the defensive role of PPO, correlations between PPO activity levels and cotton bollworm weight gains and between PPO activity levels and leaf areas consumed were estimated using a population of 22 A19-3 and S-18 transgenic plants segregating for the PPO transgenes and, hence, for PPO activities. PPO activities in these plants ranged from 1.00 to 18.95 μmol quinone formed per minute per milligram protein. At the beginning of the experiment, a leaflet from node 4 of each plant was used to measure PPO activity, and separate leaflets from the same leaf were used for the feeding assay. Neonate larvae were allowed to feed on detached node 4 leaves of individual plants (ten neonates per leaf) for 2 days. Then, five larvae were randomly selected, weighed, and divided onto detached node 4 leaflets of the same plant, the leaf areas of which had been measured. One larva was placed onto each leaflet in an individual plastic container (6-cm diameter). Larvae were allowed to feed on leaves for 7 days. Leaves whose petioles were wrapped in wet cotton were changed every 2 days, or sooner if they were almost completely eaten, so that larvae were never food limited. After 7 days, the weight gains and remaining leaf areas were recorded. Correlation between PPO activity levels and cotton bollworm weight gains and between PPO activity levels and leaf areas consumed were analyzed by SAS (SAS Institute 2003).

Beet Armyworm [Spodoptera exigua (Hübner)] Feeding Assays Beet armyworm larvae were collected from vegetable farms in Nakhon Ratchasima province, Thailand and reared on artificial diet until pupation. Male and female adults were allowed to randomly mate and lay eggs. Five genotypes of tomato, A14-6, A19-3, NT, S-18, and S-28, were used to evaluate the effect of PPO on larval performance of beet armyworm when their plant-derived diets varied in PPO activity levels. The experiments were conducted three times at different times of the year (experiment 1 in March, experiment 2 in October, and experiment 3 in June). After hatching, the neonates were equally divided and placed onto detached leaves of each genotype that were placed in plastic boxes (16×22 cm^2), one genotype per box. After 5 days (experiments 1 and 2) or 7 d (experiment 3), larvae were weighed and transferred to individual plastic containers (6-cm diameter; one larva

per container) containing one or more detached leaves, the leaf areas of which had been measured with a leaf area meter. Larvae were allowed to feed for 5 days (experiment 1) or 7 days (experiments 2 and 3). Leaves were changed every 2 days, or sooner if they were almost completely eaten, so that larvae were never food-limited. After 5 or 7 days of feeding, the mortality and weights of larvae and remaining leaf areas were recorded, and weight gains, RGRs, percent mortality, and leaf areas consumed were calculated as described for the cotton bollworm feeding assay. Weight gains were calculated at two stages of larval growth; the first stage at 0–5 days old (experiments 1 and 2) and 0–7 days old (experiment 3) and the second stage at 5–10 days old (experiment 1), 5–12 days old (experiment 2) and 7–12 days old (experiment 3). Leaf areas consumed were calculated only from the second stage in all three experiments. In experiments 1 and 2, larvae were continuously fed on foliage of the five tomato genotypes until pupation. Pupal weight was measured 24 h after pupation. Larval lifespan was calculated as the time from egg eclosion to pupation.

Fifteen replications (three larvae per replication), seven replications (two larvae per replication), and ten replications (three larvae per replication) were used in experiments 1, 2, and 3, respectively. Each larva was maintained in its own plastic container and fed leaves from three to five plants grown near each other for the entire experiment. For statistical analysis, mean weights, leaf areas consumed, and larval life spans were calculated for the two or three larvae in each replication. The mean weights, areas, and life spans for all replications were used in the analyses. For experiments 1 and 2, the effects of plant genotype on first stage weight gains, pupal weight, and larval life span were analyzed by one-way ANOVA. The effects of plant genotype on second-stage weight gains and leaf areas consumed were analyzed by ANCOVA with initial larval weight as a covariate. ANCOVA could not be used for the analysis of first-stage weight gains because neonate larvae were too small to obtain an initial weight (i.e., initial weights for neonate larvae were assumed to be zero). For experiment 3, the effects of plant genotype and leaf node on first stage weight gains were analyzed by two-way ANOVA, while two-way ANCOVA with initial larval weight as a covariate was used to analyze the effects of plant genotype and leaf node on second-stage larval weight gains and leaf areas consumed. In addition, an ANCOVA was conducted with weight gain as the response variable and leaf consumption as the covariate as described for the cotton bollworm feeding assay.

Protein Extraction, Quantification, and PPO Activity Assay Tomato leaflets (from node 4 and/or node 8) were collected at the beginning of insect feeding assays and homogenized at a ratio of 200-mg fresh weight to 1-ml homogenization buffer [0.1 M Tris–HCl, pH 7.0, 0.1 M KCl, 1 mM PMSF, 1 μg ml^{-1} leupeptin, 1% (*v*/*v*) Triton X-100, 3% (*w*/*v*) PVPP, and 1 mM Na_2EDTA). The homogenate was centrifuged at 10,000×*g* at 4°C for 20 min, and the supernatant was used for protein quantification and PPO activity assay. Total protein was quantified by the Bradford method (Bradford 1976) using bovine serum albumin as standard. PPO activity was measured spectrophotometrically following Thipyapong et al. (1995) using 2 mM 4-methylcatechol as a substrate. Catalase (280 U) was added to each sample 15 min prior to measurement for elimination of peroxidase-mediated phenolic oxidation. Specific activity was expressed as micromole quinone formed per minute per milligram protein. Seven replicates were used for each treatment. One-way ANOVA was used to evaluate the effect of plant genotype on PPO activity using SAS (SAS Institute 2003).

Results

Overexpression of PPO Increases Resistance to Cotton Bollworm The role of PPO in resistance to cotton bollworm was evaluated with a transgenic tomato line that overexpresses PPO (S-18) and two independent transgenic lines with suppressed PPO levels (A14-6, A19-3). Growth on these transgenic lines was compared to growth on the NT control with intermediate PPO level. At the time when the cotton bollworm performance was evaluated, foliage of OP plants showed 2.6-fold higher PPO activity levels than NT plants, while SP plants had 1.5- to 2.9-fold lower PPO activity levels than NT plants (Table 1). Four-day-old larvae were placed on nodes 4 and 8 leaves of tomato genotypes with varied PPO activity levels and allowed to feed for 7 days. Larval weight gains were significantly affected by plant genotype ($F_{3,29}$=6.35, P=0.002), leaf node ($F_{1,29}$=4.32, P=0.05) and by the interaction of plant genotype and leaf node ($F_{3,29}$=13.6, P<0.001). The covariate (initial weight) effect was not significant ($F_{1,29}$= 0.03, P=0.87). Weight gains were higher on node 8 leaves than on node 4 leaves, and the effects of plant genotype on weight gains were much more pronounced on node 8 leaves than on node 4 leaves. On node 8 leaves, weight gains were highest on plants with suppressed PPO activities, intermediate on wild-type plants, and lowest on lines with overexpressed PPO. The weight gains of cotton bollworm larvae feeding on node 8 A14-6 and A19-3 leaves were 3.0- and 1.9-fold higher than those feeding on NT leaves, respectively. The ANOVA of larval RGRs yielded results that were very similar to the ANCOVA analysis (data not shown).

Leaf areas consumed were significantly affected by plant genotype ($F_{3,29}$=5.85, P=0.003), leaf node ($F_{1,29}$=34.44,

Table 1 Leaf area consumption, percent mortality, and weight gains of *H. armigera* feeding on tomato leaves from genotypes differing in PPO activity levels

Leaf node	Genotype	PPO activity (μmol quinone formed per minute per milligram protein)	Leaf area consumption (cm^2)	Weight gain (mg)	RGR ($mg\ mg^{-1}\ day^{-1}$)	Percent mortality
4	A14-6[a]	0.825±0.147[b]	7.094±0.740	31.27±3.60	0.260±0.001	13.33
	A19-3	1.596±0.243	6.347±0.739	29.52±2.21	0.259±0.005	0.00
	NT	2.399±0.286	9.154±0.787	41.41±3.40	0.262±0.004	0.00
	S-18	6.323±0.903	7.234±1.130	31.34±4.10	0.254±0.009	13.33
8	A14-6		18.275±1.315	71.42±7.62	0.275±0.001	0.00
	A19-3		14.429±1.047	45.65±4.52	0.268±0.003	0.00
	NT		6.587±1.180	23.80±6.04	0.243±0.009	0.00
	S-18		8.640±1.118	24.34±5.23	0.249±0.007	7.14

RGR relative growth rate
[a] A14-6, A19-3: transgenic lines with suppressed PPO activity; NT: non-transformed control; S-18: a transgenic line overexpressing PPO activity
[b] Data are presented as means±SE

$P<0.001$), and by the interaction of plant genotype and leaf node ($F_{3,29}=17.74$, $P<0.001$). The covariate effect was not significant ($F_{1,29}=0.25$, $P=0.62$). At node 4, leaf areas consumed by cotton bollworm were not markedly different among genotypes. However, at node 8, the amount of foliage consumed by cotton bollworm differed greatly among genotypes. Cotton bollworm larvae feeding on A14-6 and A19-3 leaves consumed 2.2 to 2.8 and 1.7 to 2.1 times more foliage than those feeding on NT and S-18 leaves, respectively.

When the effects of plant genotype on weight gains of larvae were further analyzed using leaf consumption as a covariate, the covariate effect was significant ($F_{1,27}=16.34$, $P<0.001$), but the effects of plant genotype, leaf node, and leaf node by genotype were not (genotype, $F_{3,27}=1.92$, $P=0.15$; node, $F_{1,27}=1.82$, $P=0.19$; genotype by node, $F_{3,27}=1.59$, $P=0.21$).

Finally, when cotton bollworm neonates were fed, in a separate experiment, on node 4 leaves of a population of transgenic tomato plants segregating for PPO activities (activities ranged from 1.00 to 18.95 μmol quinone formed per minute per milligram protein), a negative correlation was found between PPO activity levels and weight gains ($r=-0.598$, $P<0.001$; $y = 3.0764x + 55.421$). Similarly, PPO activity levels were negatively correlated with foliar consumption ($r=-0.540$, $P<0.001$; $y = -0.4859x + 11.618$; Fig. 1).

Overexpression of PPO Increases Resistance to Beet Armyworm PPO-mediated resistance to another tomato Lepidopteran insect pest, *S. exigua*, was also evaluated in three experiments with five tomato genotypes [two independent SP lines (A14-6 and A19-3), 2 independent OP lines (S-18 and S-28), and NT control] in a manner similar to that used with *H. armigera*. In experiments 1 and 3 (PPO activities were not determined in experiment 2), differences in PPO activity levels were found among NT, OP, and SP plants. The lines S-18 and S-28 had the highest PPO activity (1.7- to 4.5-fold higher than NT), NT had intermediate PPO activity, and A14-6 and A19-3 had the lowest PPO activity (1.3- to 3.1-fold lower than NT;

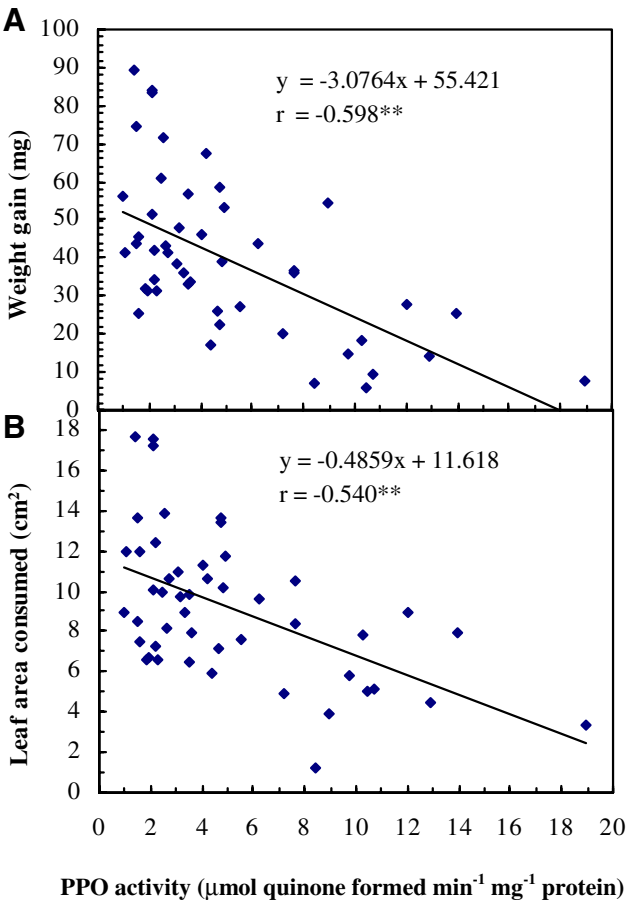

Fig. 1 Correlation between PPO activity and (**a**) weight gain or (**b**) leaf area consumption of cotton bollworm larvae (*H. armigera*) feeding on node 4 leaflets of transgenic tomato plants segregating for PPO activity levels

Table 2). Similar variation in immunologically detectable PPO levels among tomato genotypes also was observed in experiment 2 (data not shown). In agreement with previous reports (Thipyapong et al. 2004a, b; Mahanil et al. 2008), basipetal declines in PPO activities from younger (node 4) to older (node 8) leaves were observed in all five tomato genotypes (Table 2).

The effects of PPO over- and underexpression on *S. exigua* feeding and growth were similar to those observed for cotton bollworm. Consumption of foliage by beet armyworm larvae significantly differed when feeding on different tomato genotypes in both experiment 1 (leaf node 8) and experiment 2 (leaf node 4; experiment 1, main effect of genotype, $F_{4,66}=8.66$, $P<0.001$; effect of covariate, $F_{1,66}=6.80$, $P=0.01$; experiment 2 main effect of genotype, $F_{4,24}=3.33$, $P=0.03$; effect of covariate, $F_{1,24}=0.34$, $P=0.57$). In experiment 3, leaf areas consumed by larvae were significantly affected by leaf node ($F_{1,76}=3.84$, $P=0.05$), but the effect of plant genotype was only marginally significant ($F_{4,76}=2.39$, $P=0.06$), and the genotype by node effect was not significant ($F_{4,76}=0.76$, $P=0.55$). The effect of initial weight (covariate) in experiment 3 was significant ($F_{1,76}=27.99$, $P<0.001$). OP plants with the highest PPO activity levels had the lowest leaf areas consumed in all three experiments at both young (node 4) and old (node 8) leaves. In contrast, consumption of foliage on SP plants with the lowest PPO activity levels was significantly higher than consumption of foliage on NT and OP plants in most experiments. In experiment 1, leaf areas consumed by beet armyworm were the lowest on OP plants (2.1 to 3.5 times lower than SP and 1.5 to 2.2 times lower than NT plants, respectively). In experiment 2, larvae on A19-3 consumed more leaf area than larvae on NT (1.4 times) and OP (2.3 to 2.4 times) plants. In experiment 3, foliar consumption at node 4 of OP plants were 1.6 to 2.6 and 1.8 to 3.1 times lower than foliar consumption on NT and SP plants, respectively. At node 8, the consumption of OP foliage was 1.9 to 2.2 times lower than consumption of A14-6 foliage (Table 2).

Weight gains were measured at two stages. The first stage encompassed days 0 to 5 after initiation of feeding in experiments 1 and 2 and days 0 to 7 in experiment 3. The second stage encompassed days 5 to 10 after initiation of feeding in experiment 1, days 5 to 12 in experiment 2, and days 7 to 12 in experiment 3. First-stage weight gains differed significantly among tomato genotypes in experiment 1 (leaf node 8) and experiment 2 (leaf node 4; experiment 1: $F_{4,67}=109.23$, $P<0.001$; experiment 2: $F_{4,26}=17.86$, $P<0.001$). First-stage weight gains in experiment 3 were significantly affected by genotype and node, but not by the interaction of genotype and node (main effect of genotype, $F_{4,90}=21.45$, $P<0.001$; main effect of node, $F_{1,90}=4.88$, $P=0.03$; genotype by node, $F_{4,90}=2.34$, $P=0.06$). In all experiments, first-stage weight gains of beet armyworm larvae feeding on OP leaves were lower than those of larvae feeding on SP leaves. The differences were 1.4- to 3.3-fold. Also, beet armyworm larvae feeding on SP leaves possessed higher first-stage weight gains than those feeding on NT leaves (1.2- to 2.4-fold). In experiment 1, feeding on OP leaves resulted in 1.7- to 1.9-fold reduction in first-stage weight gains, while feeding on SP leaves enhanced first-stage weight gains by 1.2- to 1.8-fold relative to NT plants. In experiment 2, first stage weight gains were 1.4- to 2.4-fold higher on SP leaves than on NT and OP leaves. In experiment 3, larvae feeding on SP leaves had 1.8- to 2.8-fold and 1.5- to 2.5-fold higher first-stage weight gains than those feeding on OP leaves at nodes 4 and 8, respectively.

Second-stage weight gains were similarly affected by the suppression and overexpression of PPO; larval growth differed on different tomato genotypes at leaf node 8 (experiment 1: $F_{4,66}=21.01$, $P<0.001$; $F_{1,66}=5.01$, $P=0.03$ for the covariate effect), but not leaf node 4 (experiment 2: $F_{4,25}=0.47$, $P=0.76$; $F_{1,25}=5.77$, $P=0.02$ for the covariate effect). In experiment 3, weight gains were significantly affected by plant genotype ($F_{4,76}=4.43$, $P=0.003$), leaf node ($F_{1,76}=5.01$, $P=0.03$), and initial weight ($F_{1,76}=14.10$, $P<0.001$), but not by the interaction of node and genotype ($F_{4,76}=0.52$, $P=0.72$). Rearing the larvae on OP leaves again resulted in the lowest second-stage weight gains. When compared to weight gains of larvae feeding on SP lines, the second-stage weight gains of larvae feeding on OP leaves were 1.3- to 3.7-fold lower. In experiment 1, second-stage weight gains of larvae feeding on OP leaves were 1.2- to 2.3-fold and 1.8- to 2.9-fold lower than those feeding on NT and SP leaves, respectively. Figure 2 shows that sizes of beet armyworm larvae varied when fed on node 8 leaves of plants differing in PPO activity levels in this experiment. In experiment 2, consumption of A19-3 leaves led to the highest second-stage weight gains (1.5-fold and 1.7- to 1.9-fold higher than those feeding on NT and OP leaves, respectively). In experiment 3, beet armyworm feeding on OP leaves at node 4 exhibited 1.6- to 3.1-fold and 1.9- to 3.7-fold lower second-stage weight gains than those feeding on NT and SP leaves, respectively. At node 8, the second-stage weight gains of larvae feeding on OP leaves were 2.2- to 2.8-fold lower than those feeding on SP leaves.

In the two experiments (experiments 1 and 2) in which larvae were allowed to feed on different tomato genotypes until pupation, pupal weight and duration of the larval stage were recorded. No significant variation in pupal weight was found among beet armyworms fed the different genotypes. However, in experiment 1, beet armyworms feeding on SP leaves tended to have higher pupal weights than those feeding on NT and OP leaves (Table 2). For larval life

Table 2 Leaf area consumption, percent mortality, weight gains, pupal weights, and durations of larval stages of *S. exigua* feeding on tomato leaves from genotypes differing in PPO activity

Experiment	Leaf node	Genotype	PPO activity (μmol quinone formed min^{-1} mg^{-1} protein)	Weight gain[a] First stage (mg)	Leaf area consumed (cm^2)	Weight gain[a] Second stage (mg)	RGR (mg mg^{-1} day^{-1})	Pupal weight (mg)	Life span (days)	Percent mortality
1	8	A14-6[b]	1.061±0.132[c]	4.92±0.32[d]	43.02±2.28	152.14±5.77[e]	0.374±0.002	81.94±2.77	12.33±0.24	2.22
		A19-3	1.506±0.162	7.17±0.23	49.93±2.27	145.92±6.05	0.362±0.003	78.67±3.57	12.58±0.42	0.00
		NT	3.106±0.408	4.00±0.12	31.35±2.20	120.74±6.43	0.374±0.002	71.76±3.11	13.06±0.28	2.22
		S-18	10.490±1.500	2.38±0.08	14.19±0.99	51.92±4.24	0.356±0.007	68.34±2.77	15.38±0.16	0.00
		S-28	14.059±0.988	2.13±0.09	20.73±1.50	79.70±4.41	0.377±0.002	73.16±2.70	14.19±0.13	0.00
2	4	A14-6		2.65±0.30[d]	17.11±3.16	119.98±15.45[f]	0.309±0.004	70.32±2.89	16.55±0.52	5.00
		A19-3		4.42±0.23	31.08±3.41	155.25±4.65	0.305±0.002	66.40±2.92	15.33±0.53	0.00
		NT		1.83±0.31	21.53±2.12	105.18±12.33	0.317±0.001	72.49±2.96	16.55±0.19	5.26
		S-18		1.84±0.24	13.09±2.88	82.75±15.84	0.309±0.003	65.94±3.19	17.25±0.53	15.00
		S-28		1.83±0.23	13.73±1.63	92.39±15.45	0.311±0.006	71.59±4.64	16.71±0.41	0.00
3	4	A14-6	0.288±0.143	4.96±0.60[g]	21.56±2.12	119.26±13.04[h]	0.368±0.004			6.67
		A19-3	0.311±0.046	4.87±0.42	20.79±2.41	120.03±12.67	0.365±0.003			13.33
		NT	0.904±0.202	3.91±0.40	17.93±1.04	98.03±10.56	0.369±0.003			0.00
		S-18	1.983±0.418	1.71±0.11	6.90±1.01	32.43±4.70	0.350±0.010			13.33
		S-28	1.525±0.146	2.81±0.27	11.27±0.55	64.32±7.81	0.359±0.004			10.00
	8	A14-6	0.162±0.039	3.46±0.46[g]	17.30±3.20	94.66±16.11[h]	0.368±0.004			3.33
		A19-3	0.123±0.058	5.11±0.56	15.77±2.98	104.24±15.12	0.359±0.006			6.67
		NT	0.209±0.036	2.70±0.22	10.37±1.39	54.11±8.74	0.359±0.005			0.00
		S-18	0.369±0.056	2.03±0.18	7.83±1.44	36.55±6.19	0.344±0.011			23.33
		S-28	0.388±0.092	2.30±0.27	9.10±1.37	40.44±5.07	0.358±0.006			0.00

RGR relative growth rate

[a] Weight gains in mg; weight gain during "first stage" of larval growth is also the initial weight for the "second stage"

[b] A14-6, A19-3: transgenic lines with suppressed PPO activity; NT: non-transformed control; S-18, S-28: transgenic lines overexpressing PPO activity

[c] Data are presented as means±SE

[d] Weight gained during 0–5 days

[e] Weight gained during 5–10 days

[f] Weight gained during 5–12 days

[g] Weight gained during 0–7 days

[h] Weight gained during 7–12 days

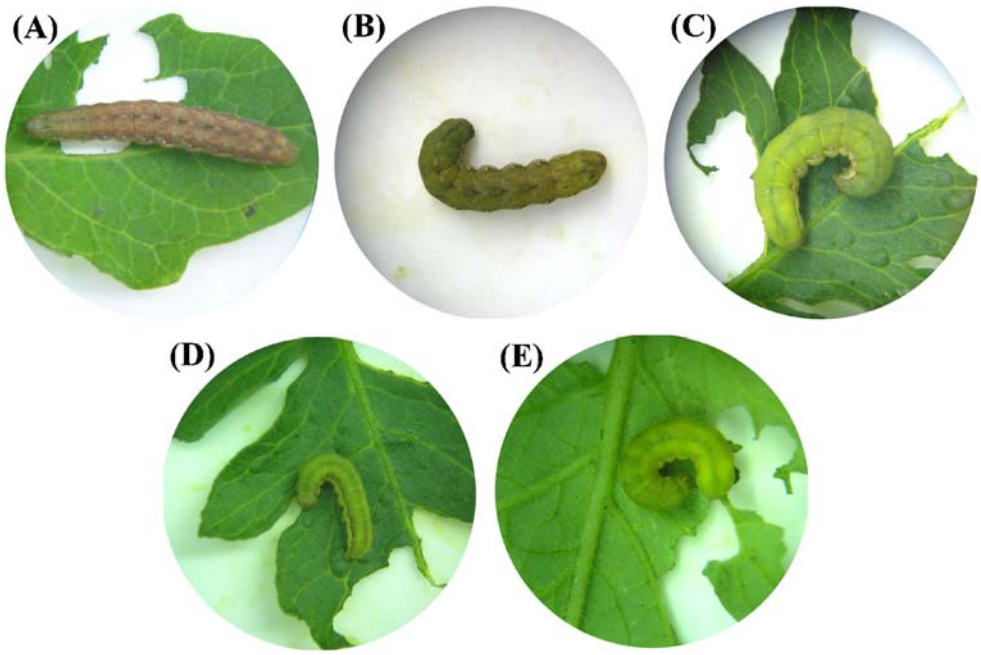

Fig. 2 Ten-day-old larvae of beet armyworm feeding on node 8 tomato leaves with differing PPO activity levels, A19-3 (**a**), A14-6 (**b**), NT (**c**), S-18 (**d**), and S-28 (**e**). *A14-6, A19-3* transgenic lines with suppressed PPO activity; *NT* non-transformed control; *S-18*, *S-28* transgenic lines overexpressing PPO activity

spans, significant differences were found among beet armyworm feeding on different genotypes in experiment 1 ($F_{4,23}$=56.99, P<0.001) when node 8 leaves were used. The larval life spans of insects feeding on OP leaves were significantly longer than those feeding on NT and SP leaves. However, life spans in the second experiment when node 4 leaves were used were not significantly different among genotypes (Table 2).

In some experiments, the percent mortality of beet armyworm feeding on S-18 plants tended to be higher than those feeding on other genotypes (Table 2), but these differences could not be evaluated statistically.

When ANCOVAs using weight gain as the response variable and leaf consumption as a covariate were used to analyze the results of experiments 1–3, the effect of plant genotype was significant in experiments 1 and 3 (experiment 1, $F_{4,66}$=20.57, P<0.001; experiment 3, $F_{4,76}$=4.29, P=0.004), but not experiment 2 ($F_{4,\ 24}$=0.54, P=0.71). The covariate effect was significant (P<0.01) in all three analyses.

Discussion

A role for PPO in resistance to arthropod herbivores frequently has been suggested due to the correlation of insect resistance with PPO activity levels and the inducibility of PPO by arthropod herbivory (Felton et al. 1989; Castañera et al. 1996; Stout et al. 1998; Cooper et al. 2004; Ramiro et al. 2006). Two mechanisms have been hypothesized for PPO-mediated insect resistance. The first is the entrapment of small soft-bodied insects by polymerized glandular trichome exudates from type A or type VI trichomes of *Solanum* species. Ruptured trichomes of these species release oxidatively polymerized exudates that limited feeding and mobility of white flies (*Bemisia tabaci*), spider mites [*Tetranychus cinnabarinus* (Boisduval)], aphids [*Myzus persicae* (Sulzer)], and potato leafhoppers [*Empoasca fabae* (Harris)], causing the death of these insects (Stoner et al. 1968; Kisha 1981; Tingey et al. 1982; Tingey and Sinden 1982; Ryan et al. 1983; Steffens and Walters 1991). Additionally, Simmons et al. (2004) found that high densities of type VI trichomes on tomato leaves could entrap *H. armigera* neonates, causing high mortality. The second putative mechanism is the PPO-mediated reduction in nutritive quality, digestibility, and palatability of plant tissues to insects. PPO-catalyzed quinones can alkylate nucleophilic amino acids, such as lysine, histidine, cysteine, and methionine of proteins, rendering them indigestible. Growth rates of *S. exigua* were reduced when fed on artificial diet supplemented with chlorogenic acid and PPO (Felton et al. 1989). Recent research, including the present study, has attempted to substantiate the role of PPO in resistance to insects using transgenic plants that overexpress PPO (see below). Use of transgenic PPO-expressing plants has partially eliminated the confounding effects of other factors (e.g., other inducible proteins and allelochemicals).

Our results using tomato plants over- and underexpressing PPO activity provide evidence (albeit not unequivocal) for PPO-mediated resistance to two Lepidopteran pests of tomato, cotton bollworm and beet armyworm. In all experiments with beet armyworm that used neonate larvae (first-stage weight gains), a significant effect of PPO (plant

genotype) was found on weight gains. Significant effects of plant genotype on larval weight gains and consumption were also found for older (second stage) beet armyworm larvae in two of three experiments. In addition, higher PPO levels were associated with longer larval development times, although no PPO effect was found on pupal weight. In general, the effects of plant genotype on beet armyworm consumption and growth were more evident when assays were conducted with older (node 8) rather than younger (node 4) leaves.

For cotton bollworm assays, which used 4-day-old larvae, suppression of PPO in node 8 leaves of SP plants allowed cotton bollworm larvae to grow 1.9 to 3.0 times faster and consume 2.1- to 2.8-fold more leaf material than larvae feeding on node 8 leaves of NT and OP plants. In contrast, significant differences in growth or consumption of bollworm larvae were not found when leaves from node 4 were used. However, when neonate bollworm larvae were used for a correlation analysis, PPO activity levels of node 4 leaves were negatively correlated with both weight gains and leaf consumption of cotton bollworm. Taken together, the results of these experiments with cotton bollworm and beet armyworm suggest that PPO exerts stronger effects on younger larvae than on older larvae and that the effects of PPO are stronger in older tomato leaves than in younger leaves.

The results presented here are in general agreement with those reported in other studies that used plants with altered PPO activities. Wang and Constabel (2004) found that forest tent caterpillar larvae feeding on leaves of transgenic poplar overexpressing PPO had reduced average weight gains and higher mortality rates than those feeding on control leaves, but only when larvae from older egg masses were used. In tomato, feeding common cutworm larvae with leaves of transgenic tomato plants overexpressing PPO also reduced their growth rates and increased their mortality compared to those feeding on leaves of NT and transgenic plants with suppressed PPO activity (Thipyapong et al. 2006; Mahanil et al. 2008). This previous work on common cutworm also showed an instar-dependent PPO effect and a lack of effect on pupal weight (Mahanil et al. 2008). Recently, the effect of induced PPO and proteinase inhibitor (PI) accumulation on growth reduction of *H. armigera* also was found in transgenic tobacco overexpressing *Tobpre-proHypSys-A*, which encodes a hydroxyproline-rich glycopeptide systemin precursor protein (Ren and Lu, 2006). However, elevated PPO levels do not always lead to reduction in the growth of insects; Barbehenn et al. (2007), for example, found only limited impact of elevated PPO activities on two lymantriid caterpillars, *Lymantria dispar* and *Orgyia leucostigma*, in transgenic poplar. Clearly, the effects of elevated PPO on insect growth and development vary according to both plant and herbivore species (Barbehenn et al. 2007; Mahanil et al. 2008).

These results implicate PPO as a component of constitutive and/or inducible defensive systems against Lepidopteran insect pests in tomato. The mechanisms through which PPO exerts its effects on insect growth are, however, still unclear. An ANCOVA of the effect of diet treatment on insect weight gains using consumption as a covariate can sometimes be used to help disentangle pre-ingestive effects (e.g., feeding deterrence) from post-ingestive effects (e.g., antibiosis; Horton and Redak 1993). The results of such an analysis of our experiments suggest that differences in the growth of beet armyworms and cotton bollworms on diets with elevated PPO activities were due at least in part to post-ingestive effects, since the use of consumption as a covariate in the analyses of weight gains failed to eliminate the treatment (plant genotype) effect on weight gains in two of three cases in which an effect of genotype was originally detected. Note also that the differences in consumption rates in our studies are not only consistent with a pre-ingestive effect (e.g., feeding deterrence) of PPO but are also with a compensation hypothesis—larvae feeding on OP leaves increased consumption of a presumably low-quality diet (stemming from the antinutritive effect of PPO) relative to that on more nutritious diets (SP and NT leaves). The latter hypothesis is supported by our previous work on dietary utilization by common cutworm larvae showing that larvae feeding on OP leaves possessed higher relative consumption rates compared to those feeding on NT and SP leaves (Mahanil et al. 2008).

For the post-ingestive effect of PPO, it is possible that the highly hydrophilic quinones generated as a result of cellular decompartmentalization by cotton bollworm and beet armyworm feeding undergo a variety of secondary reactions, forming ROS under the acidic conditions in plant tissues (Guyot et al. 1995, 1996). This reaction could be substantial in tomato where abundant PPO and phenolic compounds are stored in trichomes whose rupture could rapidly generate large amount of quinones before reaching, or while in, the low-oxygen-level midgut, as has been suggested by Barbehenn et al. (2007). Interestingly, it has been shown previously that OP tomatoes accumulated much more PPO in trichomes than NT tomatoes, whereas SP tomatoes had minimal PPO in their trichomes (Thipyapong et al. 2004b). The resulting ROS as well as quinones may be directly toxic to the arthropods. Quinones may also cross-link with nucleophilic groups of amino acids and proteins, especially the sulfhydryl, amine, and amide groups, thus leading to reduction in nutritive quality and digestibility of dietary proteins and palatability of leaf tissues (Felton et al. 1989). In particular, the alkaline pH conditions (7.5–10) of many Lepidopteran insect midguts may favor covalent bond formation and cross-linking between quinones and amino acids/proteins, rendering them unusable by digestive and absorption systems (Felton et al.

1989; Steffens et al. 1994). Moreover, quinones may damage DNA directly by covalent modification or lead to substantial alteration of DNA, lipid oxidation, and protein oxidation/fragmentation through ROS formation. ROS such as H_2O_2 could also act as a diffusible signaling molecule to induce other defense genes e.g., PIs, and genes for cellular protection and phytoalexin biosynthesis. In addition, the cross-linking of oxidized phenolics as well as oxidative cross-linking of cell wall proteins and enhanced lignin formation may contribute to additional line of defense in the form of a physical barrier that hampers nutritional acquisition by herbivores (Chamnongpol et al. 1998; Grant and Loake 2000; Orozco-Cárdenas et al. 2001). The reduction of insect growth, development, and leaf consumption observed here might result from any of these mechanisms acting alone or in combination.

It is evident that PPO-mediated resistance is effective against at least three Lepidopteran insect species in addition to a bacterial disease, *P. syringae*. However, additional field testing will be required to assess the practical usefulness of increased PPO activity levels in imparting durable, broad-spectrum resistance to tomatoes when used as a component of an integrated pest management program.

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Megalanthine, a Bioactive Sesquiterpenoid from *Heliotropium megalanthum*, its Degradation Products and their Bioactivities

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Abstract The new bioactive sesquiterpenoid (3*R*,6*E*)-2,6,10-trimethyl-3-(3-*p*-hydroxyphenylpropanoyloxy)-dodeca-6,11-diene-2,10-diol, named megalanthine, was isolated from the resinous exudates of *Heliotropium megalanthum*. The degradation products of this compound were identified. Several plant-defensive properties (insecticidal, antifungal, and phytotoxic) were evaluated after obtaining positive results in a preliminary etiolated wheat coleoptile bioassay. This bioassay showed the need to have both the phenolic and sesquiterpene moieties of the natural product present to achieve a biological effect. This result was confirmed in phytotoxicity bioassays. Megalanthine was ruled out as a significant plant–plant defense agent because of its lack of stability. The positive results recorded in the antifungal and antifeedant tests suggest, however, that this chemical is relevant in several ecological interactions involving *H. megalanthum*.

Keywords *Heliotropium megalanthum* · Resin · Sesquiterpene phenylpropanoid · Megalanthine · Etiolated wheat coleoptile bioassay · Antifeedant · Antifungal · Allelopathic assay · STS

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Introduction

Species of *Heliotropium* of the *Cochranea* section (Heliotropiaceae) are endemic to the coastal hills of northern and central Chile and southern Peru. Like many plants of that area, they characteristically produce resinous exudates that cover the leaves and stems (Johnston 1928). These exudates are associated with a complex defense mechanism that includes the prevention of excessive water evaporation and protection against UV radiation (80–320 nm), phytopathogens, and phytophagous organisms (Johnson 1983; Kelsey et al. 1984; Torres et al. 1994; Reina et al. 1997; Villarroel et al. 1997, 2001; Urzúa et al. 1998; Modak et al. 2004).

In this study, we describe the isolation and structural elucidation of megalanthine (**1**), a major component of the resinous exudates of *Heliotropium megalanthum* J. M.

Johnston (Boraginaceae), the alkaloidal fraction of which previously afforded the pyrrolizidine alkaloids (PAs) megalanthonine and lycopsamine (Reina et al. 1998).

Megalanthine spontaneously degrades. Of special interest is the comparison of the bioactivity levels of the degradation products with those of the original natural compound, since this may allow possible plant–plant ecological relationships to be established (Macías et al. 2005).

To explore the activity of the compounds, we used the etiolated wheat coleoptile bioassay, (Cutler 1984) antifeedant test, and toxic bioassays of selected compounds, against the herbivorous insect models *Spodoptera littoralis* (Boisduval), *Leptinotarsa decemlineata* (Say), and the aphid *Myzus persicae* (Sulzer), as well as antifungal studies on three *Fusarium* species. In addition, we tested the selective cytotoxicity on insect Sf9 cells derived from *S. frugiperda* pupal ovarian tissue and mammalian Chinese hamster ovary (CHO) cells. We also tested the phytotoxicity against the dicotyledonous (*Lactuca sativa*, *Lepidium sativum*, and *Lycopersicon esculentum*) and monocotyledonous (*Allium cepa* and *Triticum aestivum*) species, which are denoted as standard target species (STS) (Macías et al. 2000).

Methods and Materials

General Experimental Procedures Optical rotations were measured on a Perkin-Elmer 137 polarimeter. Infrared (IR) spectra were obtained on KBr disks on a Bruker IFS66V FTIR spectrometer. Nuclear magnetic resonance (NMR) spectra were obtained on Bruker AMX2 500 MHz, Varian UNITY-400, and INOVA-600 spectrometers with $CDCl_3$ as solvent. Chemical shifts are given in parts per million with respect to residual ^1H signals of $CDCl_3$ (δ 7.25), and ^{13}C shifts are with respect to the solvent signal (δ 77.0). High-resolution mass spectrometry was carried out on a VG AUTOESPEC mass spectrometer (70 eV). Column chromatography was performed on silica gel (63–200 mesh), and thin layer chromatography (TLC) analysis by using aluminum-packed precoated silica gel plates. For high-performance liquid chromatography (HPLC), LiChrosorb silica 60 was used in the normal-phase mode and LiChrospher RP-18 in the reverse-phase mode with a refractive index detector on a Hitachi L-6020A HPLC instrument. All solvents were spectroscopic grade or distilled from glass prior to use. Fetal bovine serum (FBS), L-glutamine, and penicillin/streptomycin were supplied by GIBCO-BRL (UK). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and *p*-nitrophenylphosphate were purchased from Sigma-Aldrich. All of these products were used as received.

Plant Material H. megalanthum J. M. Johnston was collected during the flowering season in October 1995 in the north of Chile (Atacama, III Region) and was identified by Dr. Sebastian Teiller from the Museo de Historia Natural de Santiago de Chile. A voucher specimen is deposited in the herbarium of this museum (number ST 2569).

Extraction and Isolation The resinous exudates of *H. megalanthum* were obtained by dipping 800 g of fresh plant in cold CH_2Cl_2 for 15 to 20 s. Extracts were concentrated to afford 8 g of residue. Resinous exudates were fractioned by flash column chromatography on silica gel, using CH_2Cl_2 with increasing amounts of MeOH as eluent. The major fraction was purified first by Sephadex LH-20 chromatography with *n*-hexane/$CHCl_3$/MeOH (2:1:1) as eluent and then by silica gel preparative TLC eluted with *n*-hexane/EtOAc (1:1) to give megalanthine (**1**) (300 mg, 0.0375% f.p.w.).

Megalanthine (**1**) was stored under dry conditions in the absence of light, at 4°C, but it underwent degradation under these conditions (about 20% in 1 month). Purification of the mixture with silica gel CC, eluted with a gradient of $CHCl_3$/Me_2CO, yielded a fraction with compounds of higher polarity. This fraction was found to be enriched with degradation products **2** and **3** after HPLC separation with MeOH/ACN/H_2O (2:1:2) on a RP-18 semipreparative column at a flow rate of 3 mL min^{-1} and with a refractive index detector.

Megalanthine (**1**): colorless oil; $[\alpha]^{25}_D$+28.6° (MeOH; c 0.77); IR (neat, KBr) ν_{max} cm^{-1}: 3,400 (OH), 2,973, 2,929, 1,710, 1,640, 1,614, 817; see Tables 1 and 2 for ^1H and ^{13}C NMR data; electron impact mass spectrometry (EIMS), *m/z* (rel. int.): 404 $[M]^+$ (0.5), 387 $[M–OH]^+$ (17), 386 $[M–H_2O]^+$ (14), 369 $[M–H_2O–OH]^+$ (35), 315 (2), 301 (12), 221 (20), 220 (22), 202 (32), 187 (10), 166 (20), 149 (30), 107 (100), 93 (15); high-resolution electron impact mass spectrometry (HREIMS) *m/z* calculated for $C_{24}H_{36}O_5$ 404.2563, found 404.2544.

(3R,6R,7S*,10S*)-7,10-Epoxy-2,6,10-trimethyl-3-(3-p-hydroxyphenyl propanoyloxy)-dodec-11-ene-2,6-diol* (**2**): colorless oil; $[\alpha]^{25}_D$+4.0° ($CHCl_3$; c0.26); IR (neat, KBr) ν_{max} cm^{-1}: 3,360 (OH), 2,971, 1,710, 1,616, 1,072, 920, 817; see Tables 1 and 2 for ^1H and ^{13}C NMR data; EIMS, *m/z* (rel. int.): 420 $[M]^+$ (2), 403 $[M–OH]^+$ (10), 166 (15), 149 (30), 111 (27), 107 (100), 93 (16); HREIMS *m/z* calculated for $C_{24}H_{36}O_6$, 420.2512, found 420.2500.

(3R,6S,7R*,10S*)-7,10-Epoxy-2,6,10-trimethyl-3-(3-p-hydroxyphenyl propanoyloxy)-dodec-11-ene-2,6-diol* (**3**): colorless oil; $[\alpha]^{25}_D$+11.33° ($CHCl_3$; c0.06); IR (neat, KBr) ν_{max} cm^{-1}: 3,362 (OH), 2,980, 1,714, 1,616, 1,074, 920, 817; see Tables 1 and 2 for ^1H and ^{13}C NMR data; EIMS, *m/z* (rel. int.): 420 $[M]^+$ (5), 403 $[M–OH]^+$ (15), 166 (17), 149 (26), 111 (30), 107 (100), 93 (16); HREIMS *m/z* calculated for $C_{24}H_{36}O_6$, 420.2512, found 420.2529.

Table 1 ^{1}H NMR chemical shift assignments for compounds **1**–**4** in $CDCl_3$

Position	1[a]	2[b]	3[c]	4[c]
1	1.12 (*s*)	1.14 (*s*)	1.14 (*s*)	1.14 (*s*)
3	4.74 (*dd*, *J*=10.5, 2.2)	4.78 (*dd*, *J*=9.0, 3.7)	4.78 (*dd*, *J*=10.2, 2.4)	3.33 (*dd*, *J*=10.4, 1.9)
4a	1.64 (*m*)	1.67[d]	1.78[d]	1.55 (*m*)
4b	1.56 (*m*)	1.46[d]	1.54[d]	1.39 (*dddd*, *J*=14.1, 10.4, 8.8, 5.7)
5a	1.79 (*dd*, *J*=7.8, 7.1)	1.44[d]	1.38 (*ddd*, *J*=12.4, 12.4, 4.4)	2.22 (*ddd*, *J*=13.9, 8.8, 5.6)
5b		1.24 (*m*)	1.14[d]	2.06[d]
7	5.02 (*t*, *J*=7.2)	3.77 (*dd*, *J*=7.4, 7.1)	3.77 (*dd*, *J*=7.1, 7.1)	5.20[d]
8a	2.00 (*m*)	1.79 (*ddd*, *J*=12.4, 8.7, 6.8)	1.78 (*m*)	2.06 (*m*)
8b		1.77 (*ddd*, *J*=12.4, 8.4, 5.6)		
9a	1.56 (*m*)	1.88 (*ddd*, *J*=11.8, 8.4, 6.8)	1.89 (*m*)	1.55 (*m*)
9b		1.69 (*ddd*, *J*=11.8, 8.7, 5.6)	1.54[d]	
11	5.90 (*dd*, *J*=17.3, 10.8)	5.85 (*dd*, *J*=17.3, 10.5)	5.94 (*dd*, *J*=17.3, 10.7)	5.90 (*dd*, *J*=17.3, 10.7)
12a	5.21 (*dd*, *J*=17.3, 1.2)	5.18 (*dd*, *J*=17.3, 1.6)	5.16 (*dd*, *J*=17.3, 1.2)	5.19 (*dd*, *J*=17.3, 1.3)
12b	5.06 (*dd*, *J*=17.3, 1.2)	4.98 (*dd*, *J*=10.5, 1.6)	5.00 (*dd*, *J*=10.7, 1.2)	5.05 (*dd*, *J*=10.7, 1.3)
13	1.12 (*s*)	1.13 (*s*)	1.13 (*s*)	1.18 (*s*)
14	1.52 (*s*)	1.16 (*s*)	1.15 (*s*)	1.60 (*s*)
15	1.29 (*s*)	1.28 (*s*)	1.30 (*s*)	1.27 (*s*)
2′6″	7.03 (*d*, *J*=8.5)	7.06 (*d*, *J*=8.4)	7.06 (*d*, *J*=8.6)	
3′5″	6.74 (*d*, *J*=8.5)	6.74 (*d*, *J*=8.4)	6.74 (*d*, *J*=8.6)	
7′	2.88 (*m*)	2.89 (*dd*, *J*=7.7, 7.5)	2.89 (*dd*, *J*=7.6, 7.3)	
8′	2.55 (*t*, *J*=7.7)	2.65 (*dd*, *J*=7.7, 7.5)	2.65 (*dd*, *J*=7.6, 7.3)	

Multiplicities are not repeated if identical with those in the preceding column
[a] 500 MHz
[b] 600 MHz
[c] 400 MHz
[d] Multiplicities could not be determined due to the overlap of signals

Saponification of Megalanthine Megalanthine (**1**) (20 mg) was dissolved in MeOH (0.5 mL) and 5% NaOH (3 mL) and the mixture was stirred continuously at room temperature for 12 h. After the usual workup, the product was purified by using HPLC with a silica gel analytical column eluted with $CHCl_3/Me_2CO$ (4:1) at a flow rate of 1 mL min^{-1} to yield 7 mg of dihydro-*p*-coumaric acid (**5**) and 8 mg of (3*R*,6*E*,10*S*)-2,6,10-trimethyl-3-hydroxydodeca-6,11-diene-2,10-diol (**4**).

(R)- and (S)-Methoxyphenylacetic Acid Derivatives of Compound **4** Compound **4** (4 mg) was treated with CH_2Cl_2 solutions of *N*′-dicyclohexylcarbodiimide (25 mg in 1 mL), *N*,*N*-dimethylaminopyridine (3.5 mg in 0.5 mL), and (*R*)- or (*S*)-methoxyphenylacetic acid (MPA) (10 mg in 0.5 mL); the mixture was stirred at room temperature for 22 h. Evaporation of the solvent under reduced pressure yielded a residue that was purified by HPLC with a Si 60 analytical column, eluting with $CHCl_3/Me_2CO$ (89:11) at a flow rate of 1 mL min^{-1} and a RI detector to yield 4 mg of (*R*)-MPA ester **4R** and 3.5 mg of (*S*)-MPA ester **4S**, respectively.

Preparation of **2** *and* **3** A solution of megalanthine (**1**) (60 mg) in CH_2Cl_2 (10 mL) was stirred with 1.1 equivalents of metachloroperbenzoic acid (MCPBA) at room temperature for 6 h. The crude product was purified by column chromatography $CHCl_3/Me_2CO$ (9:1). Compounds **2** (21 mg) and **3** (19 mg) were obtained after purification by HPLC by using a silica gel analytical column and eluting with $CHCl_3/Me_2CO$ (4:1) at a flow rate 1 mL min^{-1}.

Wheat Coleoptile Bioassay Wheat seeds (*T. aestivum* L. cv. Cortex) were sown on 15 cm diameter Petri dishes filled with Whatman #1 filter paper moistened with water and were grown in the dark at 24°C for 4 days. The etiolated seedlings were removed from the dishes and selected by size uniformity. Selected seedlings were placed in a Van der Wij guillotine, and the apical meristems (2 mm) were cut off and discarded. The next 4 mm of the coleoptiles were removed for bioassay and kept in aqueous nutritive buffer for 1 h to synchronize growth. Pure compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted to the appropriate concentration with a phosphate–citrate buffer containing 2% sucrose at pH 5.6 to give stock solutions with a final DMSO concentration of 0.5%. The buffer with 0.5% DMSO was used as a control. Assays were carried out in duplicate.

Test concentrations were obtained by dilution. The bioassay was performed in 10 mL test tubes: five

coleoptiles were added to each test tube containing 2 mL of the test solution. Three replicates were made for each test solution, and the experiments were run in duplicate. Test tubes were placed in a roller tube apparatus and rotated at 0.25 rpm for 24 h at 22°C in the dark. All manipulations were carried out under a green safelight. Coleoptiles were measured by digitalization of their photographic images.

Statistical Analysis Data were statistically analyzed by using Welch's test with significance fixed at 0.01 and 0.05. Results are expressed in bar charts in which the null value represents the control, negative values represent inhibition, and positive values represent stimulation of the studied parameter (Macías et al. 2000). Statistical significance is expressed by means of letters where "a" means significantly different from the control with 0.01 confidence and "b" means significantly different from the control with a confidence from 0.01 to 0.05. The absence of a letter indicates no significant difference from the control values.

Insect Bioassays *S. littoralis*, *L. decemlineata*, and *M. persicae* colonies were reared on an artificial diet (Poitout and Bues 1974), potato (*Solanum tuberosum* L), and bell pepper (*Capsicum annuum*) plants, respectively, and maintained at 22±1°C, >70% relative humidity with a photoperiod of 16:8 h (L/D) in a growth chamber.

Choice Feeding Assay These experiments were conducted with sixth instar *S. littoralis* larvae, *L. decemlineata* adults, and *M. persicae* apterous adults. Percentage feeding inhibition (%FI) indexes were calculated as described previously (Reina et al. 2001). For FI values >65%, compound **1** was tested in a dose–response experiment in order to calculate its effective antifeedant dose (EC_{50}, the effective dose for 50% feeding reduction), which was determined by linear regression analysis (%FI on log dose). The settling inhibition effect was calculated by the ratio $\%T/\%C$ where $\%T$ is the percentage of aphids settled on the treated disk and $\%C$ is the percentage of aphids settled on the control disk. Significant differences were tested by the Mann–Whitney test.

Oral Cannulation This experiment was performed with preweighed newly molted *S. littoralis* L6 larvae. A sixth instar was orally injected with 40 μg of the test compound in 4 μL of DMSO (treatment) or solvent alone (control) with a Rheodyne Hamilton syringe (50 μL) attached to a Hamilton microdispenser, as described by Reina et al. (2001). The syringe tip was inserted into the mouth of the larvae (maximum of 5 mm), and then larvae were forced to feed until no regurgitation was observed. In total, 20 larvae were "fed" this way per compound tested (Burgeno-Tapia et al. 2008). At the end of the experiments (72 h), larval consumption and growth were calculated on a dry weight basis. A covariance analysis (ANCOVA1) of food consumption (ΔI) and biomass gains (ΔB) with initial larval weight (BI) as covariate (covariate $P>0.05$) was performed to test for significant effects of the test compounds on these variables (Reina et al. 2001).

Cytotoxicity Sf9 cells derived from *S. frugiperda* pupal ovarian tissue (European Collection of Cell Cultures [ECCC]) and mammalian CHO cells (a gift from Dr. Pajares, I. C. Biomédicas, CSIC) were grown as described previously (González-Coloma et al. 2002). Briefly, Sf9 cells, derived from *S. frugiperda* pupal ovarian tissue (ECCC), were maintained in TC-100 insect cell medium supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin at 26°C. Mammalian CHO cells were grown in RPMI 1640 medium supplemented as above at 37°C under a humified atmosphere of 5% CO_2/95% air. Cells seeded in 96-well flat-bottom plastic microplates with 100 μL of medium per well (initial densities of 5×10^4 and 10^4 cells per well for the insect and mammalian cultures, respectively, were exposed for 48 h to serial dilutions of the test compounds). Cell viability was analyzed according to the MTT (Sigma) colorimetric assay method (Mosmann 1983). A 10-μL aliquot of stock MTT solution (5 mg/mL in PBS) was added to all wells, and the cultures were further incubated for 4 h. The medium was then removed by aspiration, 100 μL of dimethyl sulfoxide was added to dissolve the purple formazan precipitate, and the absorbance at 570 nm (reference wavelength of 630 nm) was measured on a microplate reader (SLT Lab Instruments, Groedig, Austria). For each treatment, cell viability was calculated as the percent absorbance of the control (untreated cells). The relative potency of **1** (EC_{50} values, the effective dose to give 50% cell viability) was determined by linear regression analysis (percent cell viability on log dose) for the sensitive cell lines.

Antifungal Activity Assays *Fusarium moniliforme* (Sheldon), *F. oxysporum* fs. *lycopersici* (Scheldt), and *F. solani* (Mart) (Spanish Collection of Type Cultures CECT codes CECT 2152, CECT 2715, and CECT 2199, respectively) were grown in potato dextrose agar in Petri dishes and kept in the dark at 27°C. These cultures were frequently renovated in order to maintain the fungus in optimal conditions. The antifungal activity of **1** was tested at several doses (0.5, 0.1, 0.05, and 0.01 mg/mL) against the three species and estimated as mycelial growth inhibition. The relative potency of **1** (EC_{50} values, the effective dose to give 50% mycelial growth inhibition) was determined by linear regression analysis (percent inhibition on dose) for the sensitive species.

Standard Target Species Bioassays Seeds of lettuce (*L. sativa* L. cv. Roman), cress (*L. sativum* L.), tomato (*L. esculentum*), onion (*A. cepa* L. cv. Valenciana), and wheat

(*T. aestivum*) were obtained from FITÓ, S.L. (Barcelona, Spain). All undersized or damaged seeds were discarded, and the assay seeds were selected for uniformity. Bioassays were carried out in 9 cm diameter plastic Petri dishes with Whatman #1 filter paper as the support.

The general procedure for seedling bioassay was as follows: 25 seeds of each species per dish, except for *T. aestivum* (ten seeds per dish), were placed in 5 mL of the test solution and incubated in the dark at 25°C. Four replicates for each concentration were set up. Germination and growth time varied for each plant species: *L. sativum*, 3 days; *L. sativa*, *L. esculentum*, and *T. aestivum*, 5 days; and *A. cepa*, 7 days.

Test stock solutions (10^{-3} to 10^{-4} M) were prepared with DMSO and then diluted to 10^{-7} M with 10 mM 2-[*N*-morpholino]ethanesulphonic acid. The following solutions were obtained by dilution while maintaining the 1% DMSO percentage. Parallel blind (1% DMSO) and positive controls were performed. The positive controls used the commercial herbicide LOGRAN, a combination of *N*-(1,1-dimethylethyl)-*N*-ethyl-6-(methylthio)-1,3,5-triazine-2,4-diamine (terbutryn, 0.6%), and 2-(2-chloroethoxy)-*N*-{[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl} benzenesulfonamide (triasulfuron, 59.4%), as an internal reference according to a previously reported study (Macías et al. 2000). LOGRAN stock solutions were prepared so that the major component (triasulfuron) was present at the same molarity as the materials to be tested.

Bioassay Data Acquisition The evaluated parameters (germination rate, root length, and shoot length) were recorded by using a Fitomed system (Castellano et al. 1999) that allowed automatic data acquisition and statistical analysis by its associated software.

Statistical Analysis Data were statistically analyzed with Welch's test with significance fixed at 0.01 and 0.05. Results are expressed in bar charts in which the null value represents the control, negative values represent inhibition, and positive values represent stimulation of the parameter under investigation (Macías et al. 2000). Statistical significance is expressed by letters with "a" meaning significantly different from the control with 0.01 confidence and "b" meaning significantly different from the control with a confidence from 0.01 to 0.05. The absence of a letter indicates no significant difference from the control values.

Results and Discussion

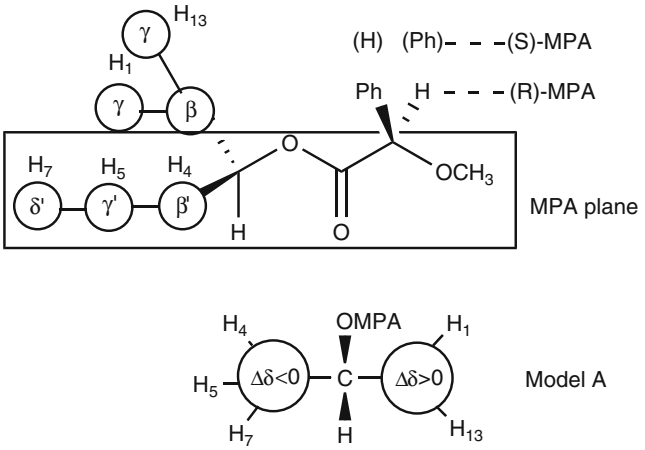
Megalanthine (**1**), the major compound of the resinous exudate (3.75%), showed a molecular ion at *m/z* 404.2544 in the HREIMS, and this is consistent with the molecular formula $C_{24}H_{36}O_5$. Additional peaks in the EIMS were observed at *m/z* 387 $[M–OH]^+$, *m/z* 369 $[M–OH–H_2O]^+$, and the base peak at *m/z* 107. A strong absorption was observed at 3,400 cm^{-1} in the IR spectrum, and this corresponds to the presence of two or more hydroxyl groups.

The ^{1}H NMR and COSY spectra (Table 1) showed signals for the protons of two subunits, a terpene and an aromatic moiety. The ^{13}C NMR spectrum (Table 2) confirmed the existence of a sesquiterpene unit with three oxygenated carbons [C-2 (δ 72.6), C-3 (δ 79.6), and C-10 (δ 73.9)] and two double bonds [C-6 (δ 134.3), C-7 (δ 125.1) and C-11 (δ 144.7), C-12 (δ111.9)]. The aromatic unit was identified as a dihydro-*p*-coumaric acid derivative. At this point, the number of unsaturations was justified and the sesquiterpene unit should be linear. Based on the structure of farnesyl sesquiterpene, the structure of **1** was proposed and this was subsequently confirmed by heteronuclear multiple bond correlations (HMBC). The position of the phenolic moiety was determined to be at C-3 because the ^{1}H NMR spectrum contained a signal at δ 4.74 *dd* for H-3, which is consistent with a vicinal acyloxy group, and a long-range correlation was observed between H-3 (δ 4.74) and C-9′ (δ 173.5) in the HMBC spectra. Compound **1** was saponified to obtain the dihydro-*p*-coumaric acid (**5**) and the sesquiterpene unit (**4**), which had the expected shielded signal for H-3 in the ^{1}H NMR spectrum (δ 3.33 *dd*). The

Table 2 ^{13}C NMR data for compounds **1**–**4** in $CDCl_3$ (100 MHz)

Position	1[a]	2	3	4
1	26.5	26.4	26.1	26.4
2	72.6	72.6[b]	72.7	73.0
3	79.6	80.2	80.6	78.2
4	27.8	23.4	23.4	29.6
5	35.9[b]	33.3	33.6	36.8
6	134.3	72.5[b]	72.5	135.3
7	125.1	85.0	85.1	125.0
8	22.7	25.9	26.0	22.7
9	41.7	37.4	37.8	41.9
10	73.9	82.9	82.6	73.5
11	144.7	143.6	144.3	144.9
12	111.9	111.4	111.7	111.8
13	24.6	25.0	24.8	23.2
14	15.8	23.9	24.0	15.9
15	27.5	26.8	26.7	27.9
1′	131.6	132.4	132.4	
2′6′	129.3	129.4	129.4	
3′5′	115.6	115.5	115.4	
4′	154.8	154.1	154.2	
7′	30.0	30.1	30.1	
8′	36.1[b]	36.1	36.0	
9′	173.5	173.1	173.0	

[a] 500 MHz
[b] These assignment may be interchanged in the same column



Δδ(R-S)	7	5	4a	4b	1	13
4	–0.33	–0.41	–0.09	–0.11	+0.21	+0.23

Fig. 1 Configurational correlation model for the (*R*)- and (*S*)-MPA derivatives. The MPA plane is shown; $H_{7,5,4a,4b}$ and $H_{1,13}$ are on the *right* and *left* sides of the plane, respectively. Model A, to determine the absolute configuration of C-3, is illustrated

spectroscopic data for the sesquiterpenic unit are in agreement with data previously described for 6*E*-2,6,10-trimethyl-3-hydroxydodeca-6,11-diene-2,10-diol (Miyase et al. 1987; Díaz et al. 1992; Miyazawa et al. 1996). Mosher's method was used to determine the absolute configuration of the chiral center (C-3) of compounds **1** and **4**. Compound **4**, which had a secondary alcohol at C-3, was esterified with the enantiomers of the chiral reagent, MPA (Latypov et al. 1996), and the resulting mixture was subjected to HPLC separation to afford compounds **4R** and **4S**.

The method requires the assignment of as many proton signals as possible of the (*R*)- and (*S*)-MPA esters, and this enables the $\Delta\delta$ (*R*–*S*) values to be determined for the protons. Protons with positive $\Delta\delta$ values should be placed on the right-hand side of model A while those with negative $\Delta\delta$ values are placed on the left (Ohtani et al. 1991) (Fig. 1). The application of the model confirmed that the absolute configuration of C-3 in compound **4** is *R*. The structure of the new sesquiterpene megalanthine (**1**) was thus determined to be (3*R*,6*E*)-2,6,10-trimethyl-3-(3-*p*-hydroxyphenylpropanoyloxy)-dodeca-6,11-diene-2,10-diol.

Compound **1** was stored under dry conditions in the absence of light at 4°C. About 20% was degraded to give two major compounds (**2** and **3**) within a month. Both compounds had very similar ^{1}H NMR spectra, and these corresponded to modifications at positions 6 and 7 of megalanthine (**1**). The H-7 signal at δ 3.77 (*dd*, *J*=7.1, 7.1 Hz) and the H-14 signal at δ 1.16/1.15 (*s*) for compounds **2** and **3** suggested oxygenation at C-6 and C-7. The ^{13}C NMR spectra of these compounds showed two signals at δ 72.5 and 85.1, which are assigned, respectively, to C-6 and C-7 by HMBC correlations. The

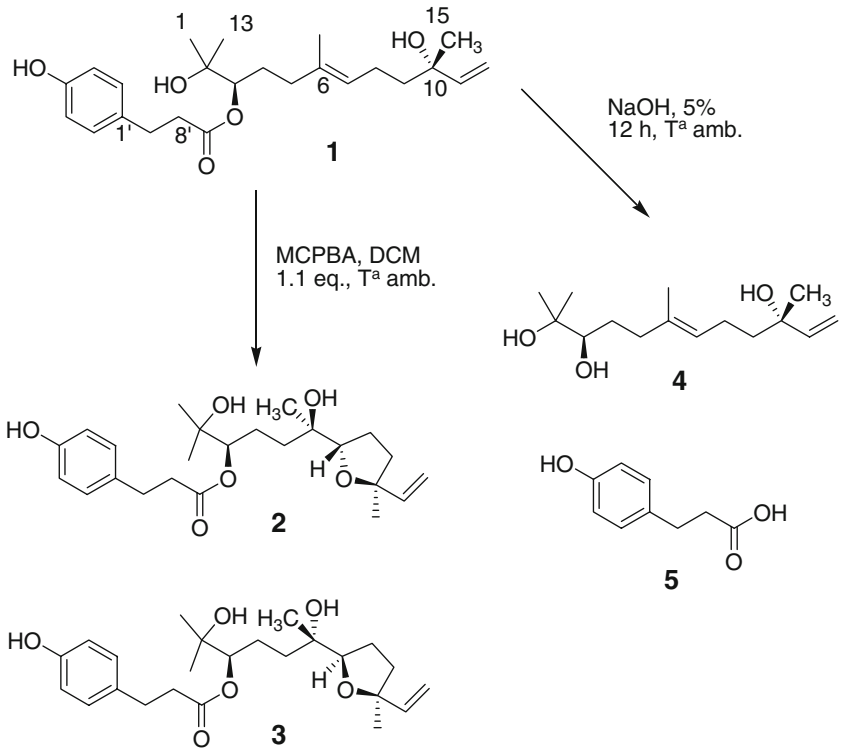
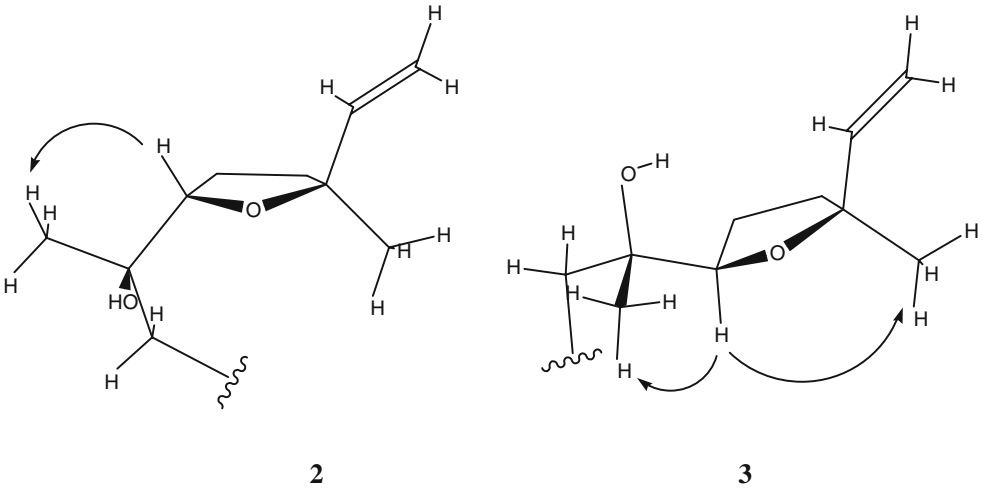


Fig. 2 Structures and chemical correlation of the new compound **1** (megalanthine), it degradation compounds **2** [(3*R*,6*R**,7*S**,10*S**)-7,10-epoxy-2,6,10-trimethyl-3-(3-*p*-hydroxyphenyl propanoyloxy)-dodec-11-ene-2,6-diol)] and **3** [(3*R*,6*S**,7*R**,10*S**)-7,10-epoxy-2,6,10-trimethyl-3-(3-p-hydroxyphenylpropanoyloxy)-dodec-11-ene-2,6-diol] and it saponification products **4** [(3*R*,6*E*,10*S**)-2,6,10-trimethyl-3-hydroxydodeca-6,11-diene-2,10-diol)] and **5** (dihydro-*p*-coumaric acid)



2 **3**

Fig. 3 NOE effects between H-7, H-14, and H-15 in the more stable conformers of compounds **2** and **3**, as identified by PM3 calculations

HREIMS data for the two compounds showed a molecular ion peak at *m*/*z* 420.2500 and 420.2529, respectively, which is consistent with a molecular formula $C_{24}H_{36}O_6$, and a base peak at *m*/*z* 107, corresponding to the *p*-hydroxyphenylmethyl radical, as in compound **1**. This molecular formula indicates the existence of an additional unsaturation in comparison to compound **1**, and this could be assigned to a cyclic ether. A study of the HMBC spectra (3 Hz) showed a correlation between C-7 and C-10, providing evidence for the presence of a tetrahydrofuranic cycle between these positions. It is proposed that compounds **2** and **3** are two isomers of (3*R*)-7,10-epoxy-2,6,10-trimethyl-3-(3-*p*-hydroxyphenylpropanoyloxy)-dodec-11-ene-2,6-diol.

The two new chiral carbons, C-6 and C-7, can generate four diastereoisomers, and the synthesis of these compounds was achieved. The first approach involved epoxidation at the C-6–C-7 double bond and ether formation by nucleophilic ring opening of the epoxide with the hydroxyl group on C-10 (Fig. 2). The reaction was carried out by using MCPBA to yield compounds **2** and **3**. Epoxide ring opening occurred under the epoxidation conditions. This reaction is an *anti* addition that provided the isomers (6*R*,7*S*) and (6*S*,7*R*).

The most stable conformer of the isomer with the relative configuration 6*S**, 7*R**, 10*S** that was found by PM3 calculations (Fig. 3) suggested an alignment of H-7 and methyl groups at 14 and 15 with the presence of a hydrogen bond between the hydroxyl group on C-6 and the tetrahydrofuranic oxygen. The nuclear Overhauser effect (NOE) experiment on **3** showed a clear effect between H-7 and the methyl groups 14 and 15, and the structure of compound **3** is thus established as (3*R*,6*S**,7*R**,10*S**)-7,10-epoxy-2,6,10-trimethyl-3-(3-*p*-hydroxyphenylpropanoyl-oxy)-dodec-11-ene-2,6-diol.

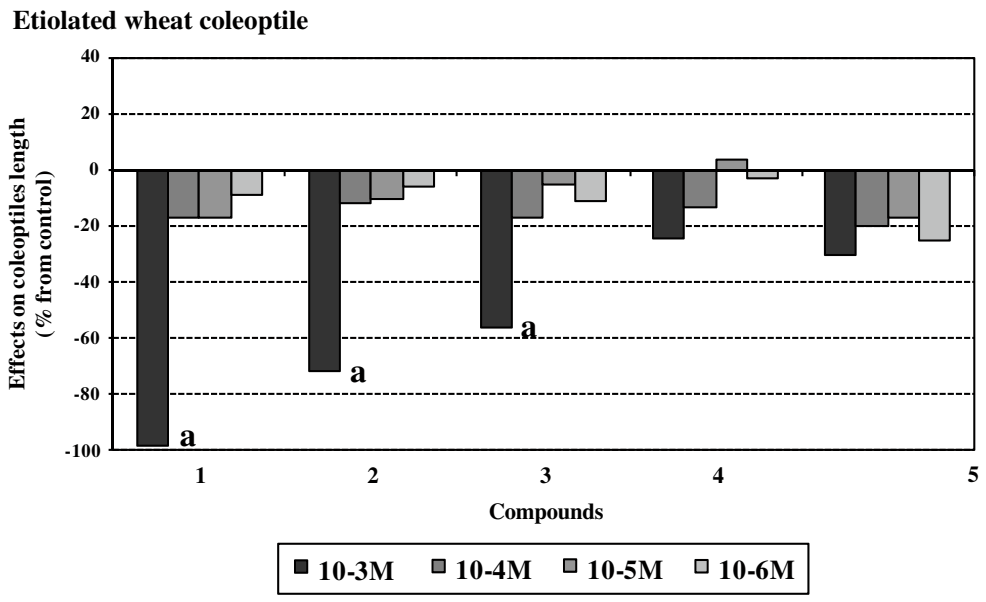


Fig. 4 Results of the etiolated wheat coleoptile assay on compounds **1**–**5**. Values are expressed as percentage differences from the control. If a letter is not indicated, then $P>0.05$ for Welch's test; *a* values significantly different at $P<0.01$

In contrast, the most stable conformer of the isomer with the relative configuration 6*R**, 7*S**, 10*S** (Fig. 3) had an alignment of H-7 and the methyl group 14 but not with methyl 15. The results of the nuclear Overhauser effect spectroscopy experiment on **2** confirmed this. The structure of compound **2** was established as (3*R*,6*R**,7*S**,10*S**)-7,10-epoxy-2,6,10-trimethyl-3-(3-*p*-hydroxyphenylpropanoyloxy)-dodec-11-ene-2,6-diol. These compounds have not been reported previously.

The first bioassay we carried out was the etiolated wheat coleoptile bioassay (Castellano 2002). This bioassay is intended to be a descriptive preliminary test. Our main objective was to perform a screening with low levels of compound, thus allowing a wider range of concentrations to be tested. This provides a general view of the possible bioactivities including plant growth regulators, herbicides (Cutler 1984; Macías et al. 2008), antimicrobials, mycotoxins, and pharmaceuticals (Jacyno and Cutler 1993).

Etiolated wheat coleoptile growth (Fig. 4) was inhibited ($P<0.01$, 98%) by a 10^{-3}-M solution of **1**. The degradation compounds **2** and **3** showed 72% and 56% of inhibition at 10^{-3} M, respectively. In contrast, the sesquiterpene and phenolic moieties (**4** and **5**) of compound **1** did not show significant activity.

The results of this screening showed that the natural product is the most active compound with the presence of sesquiterpene and phenolic moieties in the molecule as an apparent prerequisite for activity. The degradation of the compound lead to a diminution of the effect.

The phytotoxicity bioassay on STS (Macías et al. 2000) was applied to compounds **1**–**3** bearing in mind the possible degradation of compound **1** in the soil. The assay

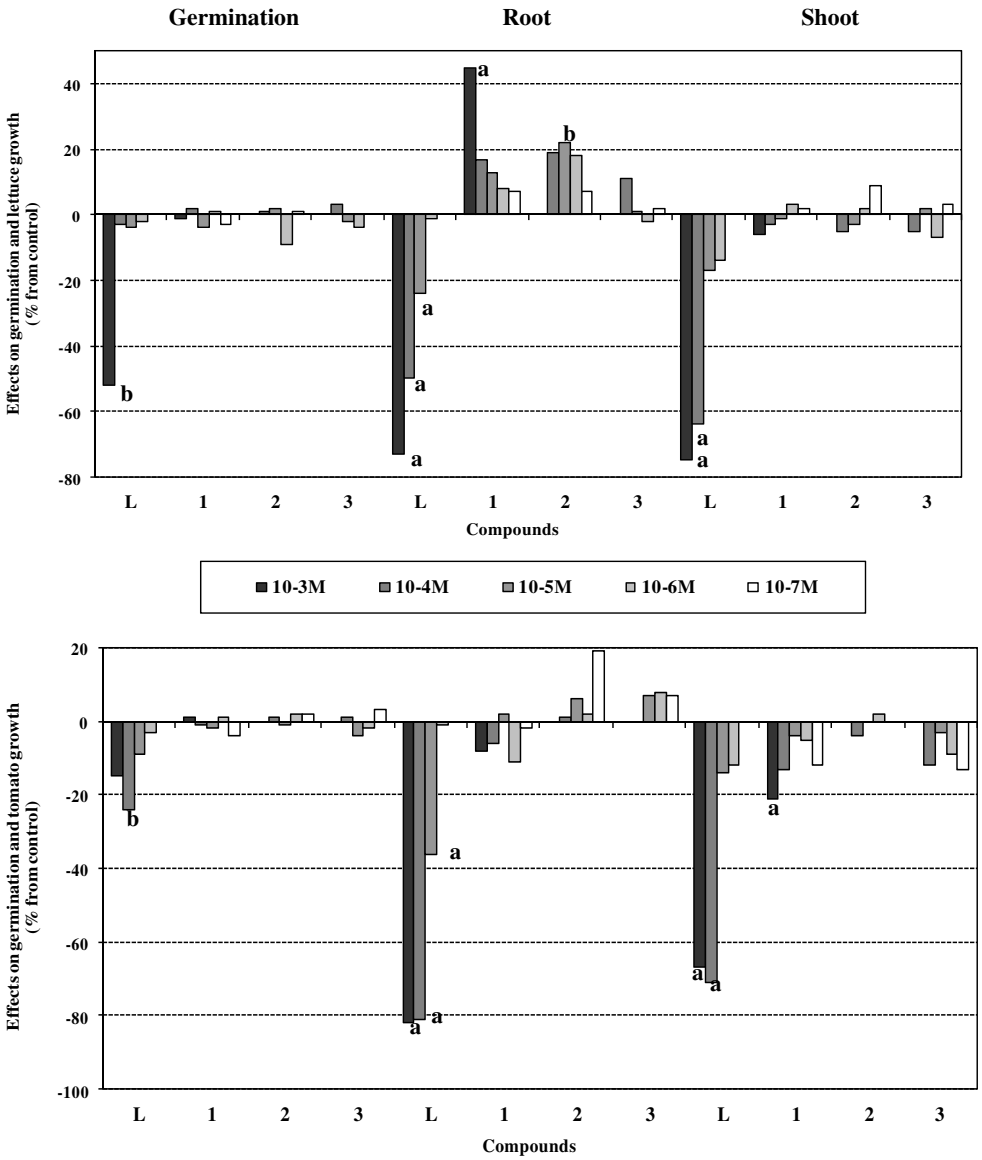


Fig. 5 Germination and growth effects of compounds **1**–**3** and LOGRAN on lettuce (*L. sativa*) and tomato (*L. esculentum*). Values are expressed as percentage differences from the control. If a letter is not indicated, $P>0.05$ for Welch's test; *a* values significantly different at $P<0.01$, *b* values significantly different at $0.01<P<0.05$

showed the effect of a series of aqueous solutions of compound **1** on the germination and growth of dicotyledonous (*L. sativa, L. sativum*, and *L. esculentum*) and monocotyledonous (*A. cepa* and *T. aestivum*) species. Data are presented as percentage differences from the control; positive values represent stimulation of the studied variable and negative values represent inhibition. The commercial herbicide LOGRAN® was used as a positive control.

Inhibition effects were not observed for lettuce (*L. sativa*), tomato (*L. esculentum*), onion (*A. cepa*), or wheat (*T. aestivum*) seeds (Figs. 5 and 6). However, there was a stimulation (average=40%) of the root growth of lettuce and wheat.

Cress seeds (*L. sativum*) were the most sensitive to the compounds tested (Fig. 7). Megalanthine (**1**) was phytotoxic to cress at 10^{-3} M, showing inhibition values of 34% for germination, 43% for root length, and 62% for shoot length. The degradation compounds **2** and **3** had reduced activity compared to compound **1**. Compounds **1**–**3** again showed a stimulation (average=40%) of the root length of cress at concentrations ranging from 10^{-4} to 10^{-7} M, as described previously for root growth of lettuce and wheat. These results suggest that megalanthine probably has no relevant role in the plant–plant defense.

In order to explore other possible roles for megalanthine (**1**), we tested megalanthine for antifeedant and antifungal effects on several target organisms. It had a moderate antifeedant effect (Table 3) on the lepidopteran *S. littoralis* without postingestive toxicity. It was a stronger antifeedant to *L. decemlineata*, but proved to be inactive to the aphid *M. persicae*. This compound was a selective cytotoxic

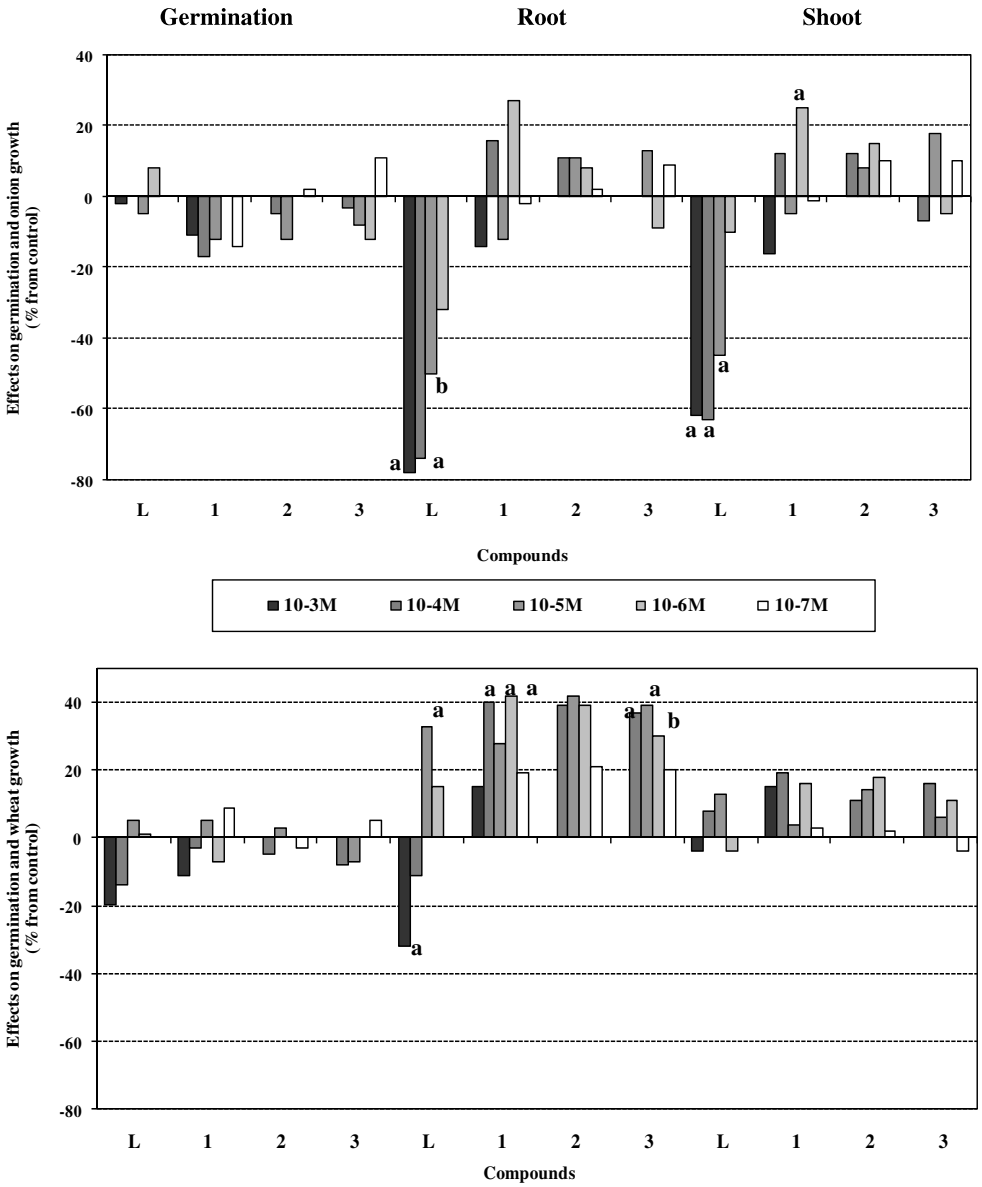


Fig. 6 Germination and growth effects of compounds **1**–**3** and LOGRAN on onion (*A. cepa*) and wheat (*T. aestivum*). Values are expressed as percentage differences from the control. If a letter is not indicated, $P>0.05$ for Welch's test; *a* values significantly different at $P<0.01$, *b* values significantly different at $0.01<P<0.05$

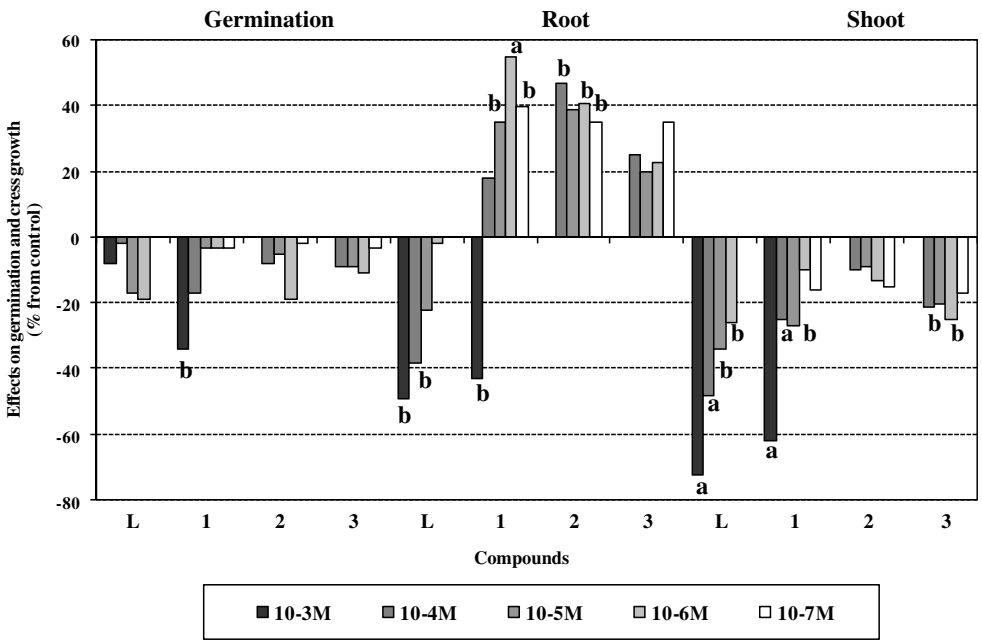


Fig. 7 Germination and growth effects of compounds **1**–**3** and LOGRAN on cress (*L. sativum* L.). Values are expressed as percentage differences from the control. If a letter is not indicated, $P>0.05$ for Welch's test; *a* values significantly different with $P<0.01$, *b* values significantly different with $0.01<P<0.05$

agent to insect-derived Sf9 cells, thus suggesting that the lack of *in vivo* toxicity on *S. littoralis* larvae could be the result of metabolic detoxification. Megalanthine (**1**) showed a significant and selective mycelial growth inhibition against the plant pathogen *Fusarium solani*.

The species-dependent antifungal and antifeedant effects with insect-selective cytotoxicity of megalanthine support a plant-defensive role against herbivorous insects and phytopathogens.

Our previous work on the alkaloidal fraction of this plant resulted in the isolation of an antifeedant with low to moderate selectivity to *S. littoralis*, the PA lycopsamine (Reina et al. 1998). However, the phenylpropanoid sesquiterpene (**1**) studied shows stronger antifeedant and antifungal action than lycopsamine. Phenylpropanoids have a multiplicity of functions in plants. The biosynthesis responds to environmental stresses such as wounding, pathogen infection, and UV radiation (Kliebenstein 2004; Dixon et al. 2002).

Table 3 Biological effects of megalanthine (**1**) on a series of biological targets related to plant defense: herbivorous insects (*S. littoralis*, *L. decemlineata*, and *M. persicae*), insect and mammalian cells (Sf9 and CHO), and phytopathogens (*Fusarium* spp.)

Target organism	Biological effects				
	Insecticidal			Cytotoxic	Antifungal
	Antifeedant	Postingestive			
		ΔB	ΔI		
S. littoralis	6.5×10^{-8} (1.8×10^{-8}, 23.2×10^{-8})[a]	109	99		
L. decemlineata	2.1×10^{-8} (1.0×10^{-8}, 4.2×10^{-8})[a]				
M. persicae	56:44[b]				
Sf9				1.09×10^{-7} (0.08×10^{-7}, 14.40×10^{-7})[a]	
CHO				≈100	
F. oxysporum					$>12.4\times10^{-4}$
F. moniliforme					$>12.4\times10^{-4}$
F. solani					9.9×10^{-4} (7.4×10^{-4}, 12.4×10^{-4})[a]

ΔB change in insect body weight (dry weight, in milligrams), ΔI food consumed (dry weight, in milligrams) expressed as a percentage of control
[a] Effective antifeedant, cytotoxic, or antifungal dose EC_{50} (in moles per square centimeter and molar)and 95% confidence limits (lower, upper)
[b] Percentage of aphids settled on treated (T) to control (C) leaf disk ratio (T/C)

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Isolation of Three Diterpenoid Acids from Sunflowers, as Oviposition Stimulants for the Banded Sunflower Moth, *Cochylis hospes*

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Abstract The banded sunflower moth (BSFM), *Cochylis hospes* Walshingham (Lepidoptera: Cochylidae) is a specialist insect, the larvae of which feed on sunflowers, *Helianthus* spp., and a few other species of Compositae. It is one of the most important pests of sunflower in the USA. Previous work on *H. annuus*, the cultivated sunflower, revealed two diterpenoids that function as oviposition stimulants for female BSFM, and that other, more polar compounds also stimulated oviposition. Using a bioassay-guided approach, we isolated three additional diterpenoids, grandifloric acid (**1**), 15β-hydroxy-*ent*-trachyloban-19-oic acid (**2**), and 17-hydroxy-16α-*ent*-kauran-19-oic acid (**3**), from polar fractions of pre-bloom sunflower head extracts. In laboratory bioassays, purified natural samples of each of these compounds stimulated oviposition by female BSFM. Structure–activity relationships of the five diterpenoids known to stimulate oviposition by female BSFM are discussed.

Keywords Grandifloric acid · 15β-hydroxy-*ent*-trachyloban-19-oic acid · 17-hydroxy-16α-*ent*-kauran-19-oic acid · Lepidoptera · Cochylidae · *Helianthus annuus*

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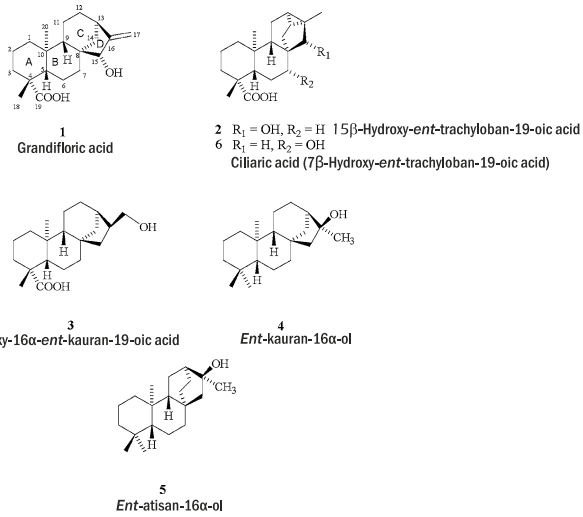
Introduction

The banded sunflower moth (BSFM), *Cochylis hospes* Walshingham (Lepidoptera: Cochylidae) is endemic to North America. Larvae of this species feed on the heads of sunflowers, *Helianthus* spp, and a few other species of Compositae. This insect is recognized as one of the most important pests of cultivated sunflower, *H. annuus* L., in the USA, particularly in the principal growing area of the northern Great Plains (Charlet et al. 1997).

As for many species of Lepidoptera, the adult female BSFM plays an important role in selection of a host on which their larvae will feed. Female BSFM oviposit predominantly on the outer whorl of involucral bracts of pre-bloom (R2-R4 stage, Schneiter and Miller 1981) sunflower heads. Upon hatching, the larvae feed initially upon bracts before moving to the disk flowers and consuming the developing seeds. Initial studies by Barker (1997) and Foster et al. (2003) indicate that nonpolar (pentane) and moderately polar (dichloromethane) extracts of sunflower bracts contain compounds that stimulate oviposition by female BSFM. Using a bioassay-driven approach, two diterpenoid alcohols, *ent* -kauran-16α-ol (**4**) and *ent*-atisan-16α-ol (**5**) were isolated from a dichloromethane extract of pre-bloom sunflower heads and found to stimulate oviposition by female BSFM. Two diterpenoid acids, *ent*-trachyloban-19-oic acid and *ent*-kaur-16-en-19-oic acid, found in relatively high abundance, also were isolated from the extract, but these compounds did not stimulate oviposition by female BSFM (Morris et al. 2005). During this work, bioassay-guided fractionation on normal-phase silica gel indicated that, apart from **4** and **5**, there were additional, more polar (later eluting) compounds that also stimulated BSFM oviposition. In this paper, we focus on the isolation and bioassay of stimulatory compounds in the more polar

fractions of pre-bloom sunflower head extracts, and report the identification of three diterpenoid acids, grandifloric acid (**1**), 15β-hydroxy-*ent*-trachyloban-19-oic acid (**2**), and 17-hydroxy-16α-*ent*-kauran-19-oic acid (**3**) that also stimulate oviposition by female BSFM.



Methods and Materials

General Fractionation and Characterization Flash column chromatography used either reversed-phase C-18 silica gel (Sigma-Aldrich, Milwaukee, WI, USA) or Diol-derivatized silica gel (Biotage, Uppsala, Sweden). Gel permeation chromatography was carried out in MeOH on lipophilic Sephadex LH-20 (Pharmacia, Uppsala, Sweden). High-performance liquid chromatography (HPLC) employed a Shimadzu LC-6A pump and SPD-10A UV detector, set to 220 nm, with an Alltech Apollo semipreparative column (5 μm C-18 reversed-phase silica, 250×10 mm) and an Alltech guard column (same media, 33×7 mm; Phenomonex, Torrance, CA, USA). Solvents used were Malinckrodt nanograde (for column chromatography), Burdick and Jackson HPLC grade (for HPLC), or redistilled AR grade. Solvents were evaporated from fractions by rotary evaporation at 40°C for larger volumes, or by a gentle stream of nitrogen for smaller volumes.

Bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% TMCS, used to trimethylsilylate acid and alcohol groups, was from Pierce (Rockford, IL, USA). Trimethylsilylation was carried out on dried subsamples in 2-ml, Teflon-capped vials, with 20–40 μl of BSTFA and 1% TMCS added. The mixture was heated for 16 h at 50°C, after which the BSTFA was evaporated under a stream of N_2, and the residue was re-dissolved in hexane for gas chromatography-mass spectrometry (GC-MS) analysis.

GC-MS was performed on a Hewlett-Packard 5890 Series II Plus gas chromatograph interfaced with a Hewlett-Packard 5792 quadrupole mass spectrometer operated (70 eV ionizing energy) in full scan mode (mass range *m/z* 35–550). A Zebron ZB-5 capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness, Phenomonex) was used for analyses, with a GC oven temperature program of 80°C (4 min hold) to 350°C at 15°C min^{-1}. Splitless injection was used, with an injector temperature of 350°C. Helium was the carrier gas, at an initial linear velocity of 30 cm sec^{-1} (constant flow setting). Mass spectra were compared with those in the Wiley Registry of Mass Spectral Data (7th Ed., John Wiley and Sons, NY, USA). Nuclear magnetic resonance (NMR) spectra were obtained on an 11.7 T Varian Inova instrument (499.67 MHz for ^1H and 125.65 MHz for ^{13}C). All spectra were recorded in $CDCl_3$ (Sigma-Aldrich). ^1H chemical shifts were referenced to residual $CHCl_3$ at 7.26 ppm, and ^{13}C shifts to the solvent signal at 77.0 ppm. The multiplicity of carbon signals was determined using DEPT experiments.

Extraction and Isolation Sunflower plants (*H. annuus* restorer line 'RHA 274') were grown in a greenhouse at 20–30°C between February and April 2004. Pre-bloom heads (R3-R4 stage, 1,651 heads, 2–3 cm diameter) were extracted in batches of 80, by dipping them into 400 ml of 3:1 MeOH/CH_2Cl_2 for 60 s, as previously described (Morris et al. 2005). The combined extracts were concentrated by rotary evaporation, leaving a MeOH solution, which was cooled to −20°C, precipitating a white waxy material. The waxy material was filtered from the extract by Buchner funnel, and the filtrate was redissolved by washing the filter paper with MeOH then CH_2Cl_2. Analysis of the filtrate by GC-MS showed it to be composed of wax aldehydes, which previously (Morris et al. 2005) had been found not to stimulate oviposition by female BSFM; therefore, this fraction was not investigated further. Celite (5 g) was added to the supernatant and the solvent removed by rotary evaporation. Rotary evaporation was stopped 5 min after apparent dryness, giving approximately 4 g dry weight of material. This material was subjected to further fractionation and bioassay.

Fractionation by reversed-phase (C-18) flash column chromatography, on 80 g of media, used a stepped solvent gradient (H_2O 200 ml, fraction 1; H_2O/CH_3CN (9:1) 200 ml, fraction 2; H_2O/CH_3CN (4:1) 200 ml, fraction 3; H_2O /CH_3CN (7:3) 200 ml, fraction 4; H_2O /CH_3CN (3:2) 200 ml, fraction 5; H_2O /CH_3CN (1:1) 200 ml, fraction 6; CH_3CN 200 ml, fraction 7; CH_2Cl_2 200 ml, fraction 8). All of these fractions were tested for the ability to stimulate oviposition by female BSFM.

Fraction 7 (624 mg) stimulated oviposition in the bioassay (see "Results" section). Therefore, an EtOH

solution of this fraction was dried onto celite (0.5 g) by rotary evaporation, then further fractionated by flash column chromatography on 25 g of Isolute Diol silica gel (hexane 150 ml, fraction 1; hexane/Et_2O (9:1) 100 ml, fraction 2; hexane/Et_2O (4:1) 100 ml, fraction 3; hexane/Et_2O (7:3) 100 ml, fraction 4; hexane/Et_2O (1:1) 100 ml, fraction 5; hexane/Et_2O (3:7) 100 ml, fraction 6; Et_2O 100 ml, fraction 7; CH_3CN 100 ml, fraction 8). Bioassay of these fractions indicated that 5 and 6 stimulated oviposition and, consequently, further effort was focused on isolating the major compounds (as detected by GC-MS) in these fractions.

Compounds **1** and **2** were isolated from Diol fraction 5 (dry weight 212 mg) by gel permeation chromatography on 50 g of Sephadex LH-20, followed by C-18 reversed-phase HPLC. Most of **1** and **2** eluted from the Sephadex LH-20 column between 160 and 190 ml of MeOH, giving 88.8 mg (dry weight). Half of this was dissolved in 500 µl of MeOH and separated by HPLC using H_2O + 0.1% *v/v* CH_3COOH/MeOH (17:83) at 5 ml min^{-1} as the mobile phase (isocratic). The peaks for **1** (7.4 min) and **2** (8.1 min) were collected as separate fractions over 23 injections resulting in 8 mg of **1** and 1.5 mg of **2**.

Compound **3** was isolated from Diol fraction 6 (dry weight 113 mg) using a method similar to that above. Fractionation on 17 g of Sephadex LH-20 gave 44.5 mg dry weight of **3** eluting between 58 and 67 ml. This was dissolved in 200 µl of MeOH and separated by HPLC as above. A peak eluting at 8.7 min was collected over ten runs giving pure compound **3** (6 mg).

Insects BSFM were obtained from a laboratory colony established in 1988 and maintained at the USDA-ARS Northern Crop Science Laboratory, Fargo, North Dakota. The colony was established from larvae collected in North Dakota and reared on a semisynthetic diet (Barker 1988). Annual addition of wild insects from North Dakota, use of R2 stage sunflower heads for oviposition in the rearing cycle, and regular tests for selectivity toward sunflower were carried out to ensure that the colony did not lose specificity to the host plant (Barker 1997). Adult BSFM were obtained <24 h after emergence and kept as mixed-sex groups (approximately 100 insects) in 8-l clear plastic, ventilated containers, at 25±1°C, 35% RH, and 16L: 8D photoperiod, for 24 to 48 h to allow mating.

Bioassay The bioassay was a modification of that described by Morris et al. (2005). Fractions, or purified compounds **1**–**3**, were applied as solutions to 10 mm diameter disks of scored chromatography paper, which were then attached to inverted glass vials (35 mm high×11 mm diameter) with a piece of mounting putty (Manco Inc., Avon, OH, USA). The disks were prepared by scoring parallel grooves, approximately 1.5 mm apart and 0.5 mm deep (seven grooves per paper disk), on a sheet of chromatography paper with a scalpel. Scoring provided physical stimulation which, along with chemical stimulation, enhances oviposition by female BSFM (Foster et al. 2003). A solution was applied to the top surface of a disk over 5 min and held by an alligator clip in the air flow of a fume hood.

Fractions 1–8 from the initial C-18 flash column fractionation of extract were taken to dryness (for weighing) then re-dissolved in solvent (H_2O for fractions 1–3, MeOH for fractions 4 and 5, MeOH/CH_2Cl_2 (1:1) for fractions 6 and 7, and CH_2Cl_2 for fraction 8). For each treatment, 50 µl [ten head equivalents (HE); one HE was the mean weight of fraction per head] of a fraction were applied to a paper disk, with 50 µl each of H_2O, MeOH, and CH_2Cl_2 for a solvent control. Vials were attached to the base of the bioassay container (17 cm diameter×14 cm high, Rubbermaid, Wooster, OH, USA) with mounting putty, and spaced evenly around the perimeter, approximately 3 cm from the wall. A replicate consisted of one treatment of each fraction plus a control, all in the same container. A circle (10 cm diameter) was removed from the lid of the arena and replaced with fine nylon mesh to allow ventilation. The arena floor was covered with moist vermiculite.

Fractions 1–8 from the Diol fractionation were tested in the same way, except all fractions were dissolved in Et_2O, and Et_2O-only solvent controls were used.

For the dose-response bioassays, purified compounds **1**–**3** were dissolved in the appropriate amount of MeOH, and 20 µl of solution were applied to paper disks to give 50, 10, and 2 µg dosages/disk; 20 µl of MeOH was used for the solvent control. HPLC-purified compounds **1**–**3** were used due to the unavailability of synthetic samples, and the difficulty of their chemical synthesis (Toyota et al. 2000). Based on the weight of these compounds recovered after Sephadex LH-20 clean-up, 50 µg is approximately one HE for compound **1**, five HE for **2**, and two HE for **3**. Replicates consisted of one treatment of each dosage of a compound, plus a MeOH-only control placed in the same container. For these experiments, vials were placed in Rubbermaid containers (14 cm diameter×11 cm high), modified as above.

Insects were sexed and placed into the bioassay containers 2–3 h before the start of scotophase. Between 15 and 30 females (24 or 48-h old), and six to ten males were used for each replicate. After five to six nights, the number of eggs on the top surface of each of the paper disks was counted. Eggs that were not laid wholly on the surface of the disks were excluded from the analysis.

Statistical Analysis Data from the bioassay of fractions were normally distributed (Shapiro-Wilk *W* test) and were

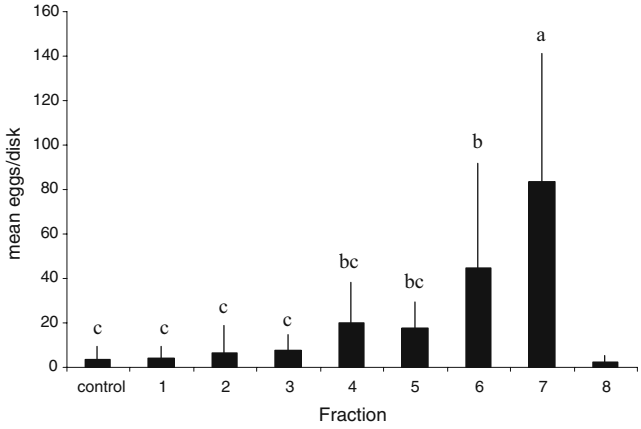


Fig. 1 Mean numbers of eggs laid by *Cochylis hospes* females on paper disks treated with fractions (ten head equivalents/fraction) from a C-18 reversed-phase silica gel flash column fractionation of an extract of 1651 R2-R4 sunflower heads. Means were calculated from five replicates. Means with different letters are significantly different at $P<0.05$, Student's *t* test. *Bars* are SE of means

analyzed by Student's *t* test using JMPin software (SAS Institute, version 4.0.4) and comparing each treatment with the control. Data from the dose-response bioassays were normal after log-transformation (Shapiro-Wilk *W* test), and analyzed by Student's *t* test, using JMPin software.

Results

Bioassay of Fractions from Sunflower Head Extract Initial fractionation of the sunflower head extract (1,651 heads) on reversed phase C-18 silica gel resulted in eight fractions that were tested in the egg-laying bioassay. Fraction 7 (eluting in 100% acetonitrile) had a greater number of eggs laid on it than on the control ($P<0.05$; Fig. 1). GC-MS analyses of TMS-derivatized fractions 5–8, showed that diterpenoids were the major class of compounds in these fractions, with angelate esters in fraction 5, diterpenoids with carboxylic acid and alcohol functional groups in fractions 6 and 7, and *ent*-trachyloban-19-oic acid and *ent*-kaur-16-en-19-oic acid in fraction 8 (previously isolated and identified by us; Morris et al. 2005).

Fraction 7 from the C-18 fractionation was further fractionated on Diol silica gel, giving eight fractions that were bioassayed. Fractions 5 and 6 [eluting in hexane/Et_2O (1:1) and hexane/Et_2O (3:7), respectively] had a greater number of eggs laid on them than were laid on the control ($P<0.05$); Fig. 2). GC-MS analyses of these two fractions (TMS-derivatized) indicated that compounds **1** and **2** were the major components of fraction 5, and compound **3** was the major component of fraction 6. Compounds **1**–**3** were purified by HPLC as described in the "Methods and Materials" section.

Identification of Compounds **1**–**3** Mass spectra of the TMS derivatives of compounds **1** and **2** {*m/z* (rel. int.): 462 (5) $[M]^+$, 447 (10) $[M\text{-}CH_3]^+$, 372 (5) $[M\text{-}(CH_3)_3SiOH]^+$, 357 (5), 332 (5), 255 (6), 254 (6), 239 (12), 198 (19), 156 (80), 73 (100)} and **2** {462 (6) $[M]^+$, 447 (8) $[M\text{-}CH_3]^+$, 372 (16) $[M\text{-}(CH_3)_3SiOH]^+$, 357 (6), 255 (7), 254 (7), 239 (7), 223 (11), 169 (23), 73 (100)} both had a molecular ion of *m/z* 462 (corresponding to *m/z* 318 before the addition of two TMS groups), suggesting they were diterpenoid acids with 5 degrees of unsaturation (rings or double bonds), similar to acids **6** and **7**, but with an additional alcohol group. Only four diterpenoids with a molecular weight of *m/z* 318 have been reported from *Helianthus* spp.; compounds **1**, **2,** and ciliaric acid, (**6**), 7β-hydroxy-*ent*-trachyloban-19-oic acid have been found in *H. annuus* (and other species) and 16β-hydroxy-*ent*-kaur-11-en-19-oic acid from *H. debilis*, *H. radula*, *H. argophyllus*, *H. ciliaris*, *H. angustifolius*, and *H. silicifolius*, but not *H. annuus* (Bjeldanes and Geissman 1972; Ohno et al. 1979; Gershenzon et al. 1981, Herz et al. 1982, 1983; Watanabe et al. 1982; Herz and Kulanthaivel 1983; Melek et al. 1985; Connolly and Hill 1991). This suggested that the compounds isolated from this *H. annuus* extract were **1**, **2**, or ciliaric acid (**6**).

Comparison of the ^{13}C NMR spectrum (Table 2) of isolated **1** with that previously reported for the 15-angeloyl ester of **1** (Ohno et al. 1979) showed that **1** was grandifloric acid. Comparison of ^{13}C NMR shifts of a number of kauranoid diterpenes (Hanson et al. 1976) indicated that the only significant effect of esterification of the 15-OH group was an upfield shift of approximately 6 ppm for C-16

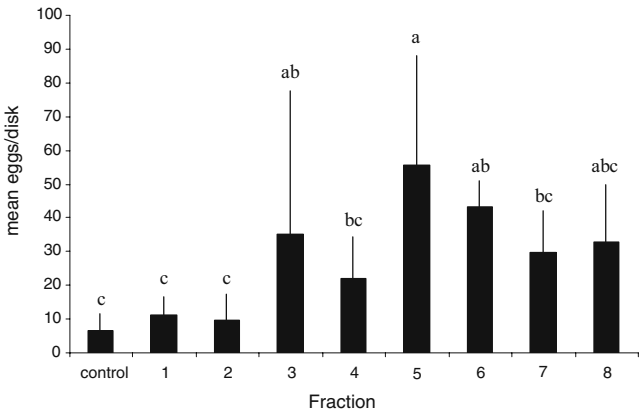


Fig. 2 Mean numbers of eggs laid by *Cochylis hospes* females on paper disks treated with fractions (ten head equivalents/fraction) from a Diol normal-phase silica gel flash column fractionation of fraction 7 in Fig. 1. Means were calculated from six replicates. Means with different letters were significantly different at $P<0.05$, Student's *t* test. *Bars* are SE of means

Table 1 Comparison of selected ^{1}H NMR chemical shifts for grandifloric acid (**1**), 15β-hydroxy-*ent*-trachyloban-19-oic acid (**2**), and 17-hydroxy-16α -*ent*-kauran-19-oic acid (**3**) isolated from sunflower head extracts, with previously reported values

Proton	Grandifloric acid (1) δ (ppm), $CDCl_3$		15β-hydroxy-*ent*-trachyloban-19-oic acid (2), δ (ppm), $CDCl_3$		17-hydroxy-16α-*ent*-kauran-19-oic acid (3), δ (ppm), $CDCl_3$	
	Measured (500 MHz)[a]	Published[b]	Measured (500 MHz)[a]	Published[b]	Measured (500 MHz)[a]	Published[c]
15	3.81 *s*	3.81 *s*	3.28 *s*	3.29 *s*	–	–
17	5.21 *s*, 5.08 *s*	5.22 *s*, 5.08 *s*	1.21 *s*	1.21 *s*	3.40 *dd* (*J*=5, 7 Hz)	3.40 *d* (*J*=7 Hz)
18	1.26 *s*	1.26 *s*	1.22 *s*	1.23 *s*	1.23 *s*	1.23 s
20	0.96 *s*	0.96 *s*	0.90 *s*	0.90 *s*	0.93 *s*	0.93 s

[a] Referenced to $CHCl_3$ at 7.26 ppm
[b] From Mullin et al. (1991)
[c] From Etse et al. (1987)

(Table 1). The minor shift difference between C-7 of **1** and C-7 of its angeloyl ester may be because of the proximity of C-7 to the –O-angeloyl group. The ^{1}H NMR spectrum of **1** was identical to that published (Table 1, Mullin et al. 1991) and very similar to that of *ent*-kaur-16-en-19-oic acid (Morris et al. 2005; Mitscher et al. 1983), except that there was a singlet at 3.81 ppm indicating a proton (H-15) on an oxygenated carbon, and the H-17 protons were shifted

Table 2 ^{13}C NMR chemical shifts for grandifloric acid (**1**), 15β-hydroxy-*ent*-trachyloban-19-oic acid (**2**), and 17-hydroxy-16α-*ent*-kauran-19-oic acid (**3**), isolated from sunflower head extracts, compared with previously reported values

Carbon	Grandifloric acid (1) δ (ppm), $CDCl_3$		15β-hydroxy-*ent*-trachyloban-19-oic acid (2), δ (ppm), $CDCl_3$		17-Hydroxy-16α-*ent*-kauran-19-oic acid (3), δ (ppm), $CDCl_3$	
	Measured (125 MHz)[a]	Published[b]	Measured (125 MHz)[a]	Published[d]	Measured (125 MHz)[a]	Published[f]
1	37.75 *t*	40.6 *t*	39.53 *t*	39.7 *t*	41.63 *t*	42.0
2	18.26 *t* [c]	19.0 *t*	18.68 *t*	18.3 *t*	19.08 *t*	19.1
3	35.09 *t*	35.1 *t*	37.77 *t*	38.4 *t*	37.20 *t*	37.2
4	43.67 *s*	43.8 *s*	43.49 *s*	43.5 *s*	44.74 *s*	44.7
5	56.94 *d*	56.6 *d*	56.43 *d*	56.6 *d*	56.98 *d*	56.9
6	19.04 *t* [c]	20.8 *t*	19.07 *t*	19.2 *t*	22.37 *t*	22.4
7	40.65 *t*	37.5 *t*	33.76 *t*	36.7 *t*	40.74 *t*	40.7
8	47.70 *s*	47.6 *s*	44.78 *s*	46.0 *s*	43.65 *s*	43.7
9	53.30 *d*	53.0 *d*	51.87 *d*	42.3 *d* [e]	56.97 *d*	56.4
10	39.77 *s*	39.9 *s*	38.66 *s*	41.2 *s*	39.61 *s*	39.6
11	20.91 *t* [c]	20.7 *t*	20.78 *t*	22.0 *t*	18.86 *t*	18.9
12	32.55 *t*	32.7 *t*	23.48 *d* [c]	20.0 *d*	31.38 *t*	31.4
13	42.27 *d*	42.6 *d*	24.95 *d* [c]	20.9 *d*	38.15 *d*	38.1
14	36.19 *t*	37.7 *t*	29.65 *t*	30.8 *t*	37.84 *t*	37.8
15	82.67 *d*	82.6 *d*	86.74 *d*	82.7 *d*	45.00 *t*	45.0
16	160.26 *s*	155.5 *s*	27.79 *s*	25.3 *s*	43.36 *d*	43.1
17	108.32 *t*	110.0 *t*	15.98 *q*	18.5 *q*	67.53 *t*	67.4
18	28.91 *q*	28.9 *q*	28.80 *q*	28.9 *q*	28.95 *q*	28.9
19	183.35 *s*	184.9 *s*	182.33 *s*	179.8 *s*	183.48 *s*	183.7
20	15.80 *q*	15.8 *q*	12.81 *q*	13.0 *q*	15.55 *q*	15.5

[a] Referenced to $CDCl_3$ at 77.00 ppm, multiplicities were determined by DEPT experiments
[b] From Ohno et al. (1979). Data for 15-angeloyl ester of **1**. Referenced to internal TMS
[c] Assignments may be interchanged
[d] From Harrigan et al. (1994).
[e] It is apparent that the data reported in Harrigan et al. (1994) for **2** from *Xylopia aethiopica* is for the 15 α-OH epimer of **2**, resulting in an upfield shift for C-9. C-9 gives a signal at 52.8 ppm in *ent*-trachyloban-19-oic acid (Beale et al. 1883), similar to that for **2** we isolated from *Helianthus annuus*. The single crystal X-ray structure of **2** from *H. annuus* shows it to have a β-OH group (Ferguson et al. 1982).
[f] From Wu et al. (1996), referenced to internal TMS.

downfield by 0.38 ppm due to being adjacent to the hydroxyl group. Signals at 5.08 and 5.21 ppm for **1** showed it had a kaurane skeleton with a double bond, rather than the trachylobane skeleton of **2** and *ent*-trachyloban-19-oic acid. The multiplicity of carbons on rings C and D (from a DEPT experiment) agreed with a kauranoid diterpene structure.

^{1}H and ^{13}C NMR spectra of compound **2** (Tables 1 and 2) showed it to be 15β-hydroxy-*ent*-trachyloban-19-oic acid, the trachylobane equivalent of **1**. Comparison of ^{13}C NMR data for **2** with that published (Harrigan et al. 1994, Table 2) showed some differences; upfield shifts for C-9, C-12, C-13, and C-15 in the published data, which all could be attributed to the compound in Harrigan et al. (1994) being the 15α-OH epimer, rather than the 15β-OH epimer. Compound **2** from *H. annuus* has been shown to be the 15β-OH epimer from a single crystal X-ray structure of its methyl ester (Ferguson et al. 1982). Comparing the ^{13}C NMR spectra of **1** and **2**, carbon signals for **1** at 160.26 ppm (singlet, C-16) and 108.32 ppm (triplet, C-17) (doubly bonded carbons) plus a triplet carbon for C-12 (32.55 ppm), were replaced in **2** by a singlet carbon at 25.3 ppm (C-16), a methyl carbon at 18.5 ppm (C-17), and a doublet carbon at 20 ppm (C-12), due to the presence of a cyclopropane ring in **2** instead of the C-16:C-17 double bond of **1**. The ^{1}H NMR spectrum of compound 2 matched published data (Mullin et al. 1991, Table 1), and showed that **2** could not be ciliaric acid (**6**), due to the ^{1}H NMR spectrum of **2** having a singlet at 3.28 ppm (H-15), instead of the multiplet at 3.64 ppm found for H-7 of **6** (Bjeldanes and Geissman 1972). A NOESY experiment showed a weak NOE crosspeak between the 3.28 ppm signal (H-15) and H-17 (1.21 ppm), indicating that the hydroxyl group was on C-15 in **2**.

The TMS derivative of compound **3** had a mass spectrum {*m/z* (rel. int.): 464 (7) $[M]^{+}$, 449 (32) $[M–CH_3]^{+}$, 374 (45) $[M–(CH_3)_3SiOH]^{+}$, 347 (55), 332 (7), 257 (27), 256 (24), 242 (20), 73 (100)} with a molecular ion at *m/z* 464, indicating that it was similar to **1** and **2**, but with one less degree of unsaturation. The ^{1}H NMR spectrum of **3** (Table 1) contained resonances for only two methyl protons, along with a doublet of doublets at 3.40 ppm (J= 5.7 Hz), suggesting a hydroxymethylene moiety, likely due to addition of H_2O across the double bond of grandifloric acid (**1**). Comparison of the ^{13}C NMR spectrum of **3** (Table 2) with those reported for the 16α and 16β epimers of 17-hydroxy-16-*ent*-kauran-19-oic acid (Etse et al. 1987; Wu et al. 1996), particularly considering the chemical shifts of C-12 and C-17, indicated that **3** was the 16α epimer, 17-hydroxy-16α-*ent*-kauran-19-oic acid, isolated previously

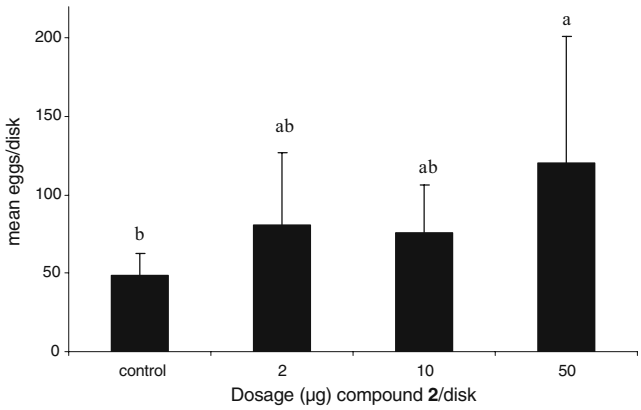


Fig. 3 Mean numbers of eggs laid by *Cochylis hospes* females on paper disks treated with different amounts of grandifloric acid (**1**). Means were calculated from six replicates, *bars* are SE of means. Means with different letters were significantly different at $P<0.05$ when log-transformed data were compared by Student's t test

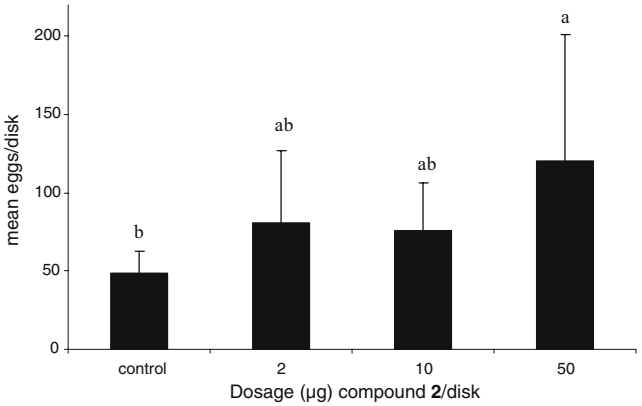


Fig. 4 Mean numbers of eggs laid by *Cochylis hospes* females on paper disks treated with different amounts of 15β-hydroxy-*ent*-trachyloban-19-oic acid (**2**). Means were calculated from six replicates, *bars* are SE of means. Means with different letters were significantly different at $P<0.05$ when log-transformed data were compared by Student's t test

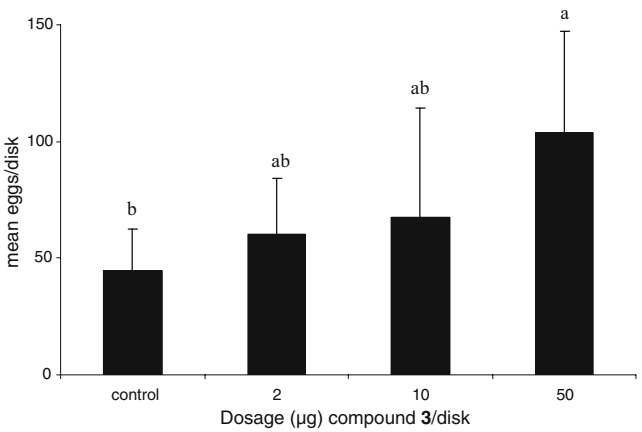


Fig. 5 Mean numbers of eggs laid by *Cochylis hospes* females on paper disks treated with different amounts of 17-hydroxy-16α-*ent*-kauran-19-oic acid (**3**). Means were calculated from five replicates, *bars* are SE of means. Means with different letters were significantly different at $P<0.05$ when log-transformed data were compared by Student's t test

from the Compositae (Moreno et al. 1980; Bohlmann et al. 1982).

The ^{1}H NMR spectra of purified **1**–**3** showed no cross-contamination of fractions, based on the methyl signals, and appeared clean of any other contamination. The absolute configuration of compound **2** from *H. annuus* is *enantio*, shown by the crystal structure of its methyl ester (Ferguson et al. 1982), and therefore, the absolute configuration of compounds **1** and **3** is also assumed to be *enantio*, as has been found for kauranoid and trachylobanoid diterpenes previously isolated from *H. annuus* (Connolly and Hill 1991). This is equivalent to (−)-kaurene and (−)-trachylobane skeletons.

Bioassay of Compounds **1**–**3** More eggs were laid on paper disks with 10 or 50 μg of grandifloric acid (**1**) than on the control (Fig. 3). 15β-Hydroxy-*ent*-trachyloban-19-oic acid (**2**) and 17-hydroxy-16β-*ent*-kauran-19-oic acid (**3**) had similar dose-response profiles (Figs. 4 and 5), with significantly ($P<0.05$) more eggs laid on paper disks treated with 50 μg of each purified compound, than on the respective controls.

Discussion

We have identified three diterpenoid acids, grandifloric acid (**1**), 15β-hydroxy-*ent*-trachyloban-19-oic acid (**2**), and 17-hydroxy-16α-*ent*-kauran-19-oic acid (**3**) from sunflower heads by using bioassay-guided liquid chromatographic fractionation. These compounds stimulate oviposition by female BSFM. In addition, two other diterpenoids, *ent*-kauran-16α-ol (**4**) and *ent*-atisan-16α-ol (**5**) (Morris et al. 2005), found in sunflower are known to stimulate oviposition by female BSFM. Comparison of the chemical structures of these five compounds shows that all five have an alcohol functional group on ring D (at positions 15, 16, or 17). This group may be a key structural feature for stimulating oviposition in female BSFM since the diterpene acids *ent*-trachyloban-19-oic acid and *ent*-kaur-16-en-19-oic acid, which do not stimulate oviposition by female BSFM (Morris et al. 2005), are structurally identical to compounds **2** and **1,** respectively, but lack the 15-OH group. The C-19 carboxylic acid group of compounds **1**–**3**, may not be important for stimulating oviposition since (inactive) *ent*-trachyloban-19-oic acid and *ent*-kaur-16-en-19-oic acid contain this group, whereas (active) **4** and **5** lack it. Other diterpenes with an alcohol group at position 16 or 17 on ring D (e.g., 16β-hydroxy-*ent*-kaur-11-en-19-oic acid) are widespread in *Helianthus*, having been isolated from at least 11 species (Gershenzon et al. 1981; Herz et al. 1982, 1983, Herz and Kulanthaivel 1983). If the structure–activity relationship above is correct, then more diterpenoids, found in a range of *Helianthus* species, could function as ovipositional stimulants for BSFM. In addition to the BSFM–sunflower system, diterpenoids have been identified as oviposition stimulants for other moth species. Duvane and labdane diterpenes (from *Nicotiana* leaves) stimulated oviposition by the tobacco budworm, *Heliothis virescens* (Jackson et al. 1986, 1991). Females of another heliothine moth, *Helicoverpa zea*, are stimulated to oviposit by two sequiterpene carboxylic acids, (+)-(*E*)-endo-β-bergamoten-12-oic acid and (+)-(*E*)- α-santalen-12-oic acid from tomato (*Lycopersicon hirsutum*) (Coates et al. 1988).

Compounds **1** and **2** have been previously isolated from *Helianthus* species, whereas compound **3** has not**,** to our knowledge. Compound **3** has been found in *Beyeria* sp. (Euphorbiacea) (Jefferies and Payne 1965), *Eupatorium tinifolium* (Compositae) (Moreno et al. 1980), and *Baccharis minutiflora* (Compositae) (Bohlmann et al. 1982). Compound **2** occurs in isolable amounts only in *H. annuus* (Connolly and Hill 1991; Gershenzon et al. 1981, Mullin et al. 1991), while compound **1**, its angeloyl ester or acid methyl ester, has been found in many species of *Helianthus* [e.g., *H. debilis* (Ohno et al. 1979), *H. radula* (Herz and Kulanthaivel 1983), *H. occidentalis* and *H. simulans* (Herz et al. 1983), *H. grosseserratus* (Gershenzon et al. 1981), and *H. niveus* (Ohno and Mabry 1980)], all of which are known hosts of BSFM (Charlet et al. 1997).

Apart from the various diterpenoid compounds discussed, the major nonvolatile components of our sunflower head extracts, identified by GC-MS, were waxes (mainly C_{20} to C_{34} even-numbered *n*-aldehydes and lesser amounts of the corresponding alcohols) and diterpene angelate esters. Sunflower head waxes do not stimulate ovipositional activity in female BSFM (Morris et al. 2005), while diterpene angelate esers were either present in too low an amount, or their fractions were relatively inactive compared with those containing compounds **1**–**3** (**1**–**3** were in fractions 6 and 7, diterpene angelate esters were in fraction 5). It is possible that esterification of diterpene alcohol groups in compounds such as **1**–**3** reduces or eliminates the ovipositional activity of the diterpenoid, especially given the structure–activity relationship discussed above.

These diterpenoids are relatively nonvolatile, and are likely perceived by female BSFM only when the female is in contact with the plant (and therefore with the diterpenoids). However, volatile compounds from sunflower are involved in host selection behavior of female BSFM (Foster et al. 2003). Future work will aim to identify these volatile chemicals.

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Phenology of Semiochemical-Mediated Host Foraging by the Western Boxelder Bug, *Boisea rubrolineata*, an Aposematic Seed Predator

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Abstract The western boxelder bug (BEB), *Boisea rubrolineata* (Heteroptera: Rhopalidae), is a specialist herbivore of boxelder trees, *Acer negundo*. We tested the hypothesis that BEBs use semiochemicals to locate host trees. Headspace volatiles from trees bearing staminate inflorescences ("staminate trees") and from trees bearing pistillate inflorescences ("pistillate trees") were collected throughout the season and bioassayed in Y-tube olfactometer experiments. Headspace extracts of early-season, pollen-bearing staminate trees and midseason pistillate trees with mature samaras (seed pods) attracted female and male BEBs. By using coupled gas chromatographic-electroantennographic detection and gas chromatography-mass spectrometry, we identified and tested a five-component synthetic blend of candidate semiochemicals (hexanol, pentyl acetate, phenylacetonitrile, 2-phenethyl acetate, and *trans*-nerolidol). This blend attracted females, males, and fifth-instar nymphs. Phenylacetonitrile by itself was as attractive as the five-component blend to both adults and nymphs. By responding to phenylacetonitrile emitted by pollen-bearing staminate trees and pistillate trees with maturing seeds, BEBs appear to track and exploit the availability of nutrient-rich food sources, suggesting that the bugs' reproductive ecology is synchronized to the phenology of their host boxelder tree.

Keywords Boxelder bugs · Rhopalidae · Heteroptera · Boxelder trees · *Acer negundo* · Semiochemicals · Phenylacetonitrile · Plant defense · Seed predation · Aposematism · Host foraging

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Introduction

It is well documented that the relationship between heteropteran phytophagous insects and their hosts is governed by ephemeral resources (Carroll 1988; Grimm and Führer 1998; Carroll et al. 2005; Kohno and Bui Thi 2005) such as pollen and seeds. Adaptations of western boxelder bugs (BEBs), *Boisea* (formerly *Leptocoris*) *rubrolineata* (Heteroptera: Rhopalidae), to plants with ephemeral resources appear diverse and may include recognition of plant semiochemicals and timing of breeding season according to resource availability. Indeed, the evolutionary trend in heteropteran true bugs from generalist predators to phytophagous specialists may be driven by interactions with host plants (Cobben 1978), thus leading to synchrony of the plants' and insects' reproductive phenologies (Aldrich et al. 1979; Bowers 1990).

The basic biology of BEBs is poorly understood. BEBs belong to the scentless plant bugs (Aldrich et al. 1990b) and feed primarily on seeds of boxelder maple, *Acer negundo* (Robinson 1996). When the weather warms in the spring, adults emerge from diapause and feed on fallen seeds (Tinker 1952) and dead insects in the soil, flowering buds in the canopy of pistillate trees, as well as anthers and associated tissues of staminate trees (JJS, unpublished data). Females deposit eggs on or near pistillate trees.

First-generation nymphs usually are found on low vegetation near trees. They reach adulthood in about 60 days (Smith and Shepherd 1937) by early to midsummer, feeding heavily on maturing seeds of pistillate trees (Long 1928; Tinker 1952). During the summer, a second, overlapping generation may emerge. By late summer into the fall, non-mating adults cease feeding, depart from senescing host trees (Tinker 1952), and form large aggregations in protective shelters for overwintering diapause.

The related and well-studied soapberry bugs, *Jadera haematoloma*, assemble on seed-bearing specimens of their host tree goldenrain, *Koelreuteria elegans* (Carroll et al. 1998). Feeding by *J. haematoloma* (Carroll 1988) or *Jadera obscura* (Tanaka and Wolda 1988) on their respective host trees attracts male and/or female conspecifics, likely mediated by semiochemicals that originate from the bugs, the plant, or both. Emission of herbivore- or wound-induced semiochemicals or semiochemicals associated with a particular stage of the host's phenology may also attract BEBs to boxelder host trees. If mate choice is tied to host choice (Feder et al. 1994; Carroll et al. 1998; Linn et al. 2004), then an assembly cue may facilitate feeding and mating during host colonization, thus driving host-specific foraging by BEBs.

If BEBs do indeed forage in response to the presence of ephemeral and highly nutritional pollen and seed resources, BEBs should be capable of exploiting semiochemical cues that might be associated with these resources. We tested the hypotheses that (1) BEBs are attracted to volatiles of early-season, pollen-bearing staminate trees but not to those of pistillate trees; (2) profiles of volatile chemicals of pistillate trees change as samaras mature and senesce; (3) BEBs discern changing volatile blends of pistillate trees and are attracted only to those associated with mature samaras (encasing maturing seeds); and (4) specific semiochemicals mediate attraction of BEBs to boxelder trees with pollen or seed resources.

Methods and Materials

Collection and Maintenance of Experimental Insects Late-instar nymph and adult BEBs were field-collected weekly on or near pistillate trees of *A. negundo* or *Acer saccharinum* in Kelowna, Westbank and Princeton, British Columbia (Canada) during June to September 2005, 2006, and 2007. BEBs were maintained at 22–30°C, 30–70% relative humidity, and a natural photoperiod. They were provisioned with water and new-growth shoots, with intact leaves and seeds, of *A. negundo* that were clipped weekly at insect collection sites (see above) and transported to Simon Fraser University (SFU) in 19-l buckets of water. Post-diapause, adult BEBs of unknown age were field-collected in March/April, separated according to sex, maintained on water in separate cages, and used in bioassays as needed. Newly molted, sexually immature adult females or males were kept separate and bioassayed within 14 days, before they reached sexual maturity (Smith and Shepherd 1937). In 2007, newly molted adults were bioassayed when 4–6 days old, an age at which long-lived seed predators feed most heavily prior to reaching sexual maturity (Caldwell and Rankin 1974). Reproductive adults (as evidenced by mating activities) were >1 month old when used in bioassays.

Collection of A. negundo volatiles Clipped branches (four to six ramates) with (1) intact leaves and reproductive parts devoid of apparent insect or mechanical damage, (2) BEBs feeding on them, or (3) mechanical damage were placed in separate water beakers (200 ml) to allow for transpiration and enclosed in separate cylindrical Pyrex® glass chambers (each 14×37.5 cm). The above treatments were chosen to discriminate between effects caused by mechanical damage or BEB herbivory. Ramates were from four different sources: (1) early-spring, pollen-bearing staminate trees or pistillate trees with swollen and opening buds, unfurling leaves, and inflorescences; (2) midspring pistillate trees with developing samaras (encasing ovules) and unfurled leaves; (3) midsummer pistillate trees with maturing seeds; and (4) late-summer pistillate trees with senescing foliage and ripened seeds.

We simulated BEB-induced mechanical damage by puncturing leaf veins, petioles, and samara ovaries with a 000-pin: each leaf received three to four vein punctures, each petiole two to three punctures, and each samara two punctures. We studied the effect of herbivory by allowing 25 3-day-starved male and 25 3-day-starved female BEBs to feed on intact branch clippings housed in a cylindrical Pyrex® glass chamber (14×37.5 cm). Charcoal-filtered air was drawn for 3 days at ~0.5 l min^{-1} through the chamber and a Pyrex® glass tube (14×1.3 cm OD) containing 500 mg of Porapak-Q (50–80 mesh, Waters Associates, Inc., Milford, MA, USA). Volatiles were eluted from the Porapak-Q with 2 ml of redistilled pentane, and extracts were concentrated as needed under a stream of nitrogen. The dry weight of ramates with attached foliage was obtained to determine gram-hour-equivalents (GHE; 1 GHE=volatiles released from 1 g of ramate biomass during 1 h), a measurement of the stimuli tested in bioassays. The procedures for obtaining control stimuli were identical to those described above, except that aeration chambers did not contain insects or plant material.

Analyses of Extracts Aliquots of headspace (above sample) extracts were analyzed by coupled gas chromatographic-electroantennographic detection (GC-EAD; Gries et al., 2002), employing a Hewlett Packard 5890A gas chromatograph equipped with a DB-5 (J&W Scientific, Folsom, CA,

USA) column (30 m×0.25 ID), helium as carrier gas (35 cm s^{-1}) and with the following temperature program: 50°C (2 min), then 10°C min^{-1} to 280°C (5 min). GC-mass spectrometry (MS) analyses of compounds that (1) elicited responses from antennae of male or female BEBs and (2) varied in abundance as the season progressed employed a Saturn 2000 Ion Trap (Varian) fitted with the DB-5 column. Compounds (Table 1) were identified by comparing

Table 1 List of antennal stimulatory components in gas chromatographic-electroantennographic detection analyses from headspace extracts of pistillate boxelder trees, *A. negundo*, with developing ovules and maturing seeds

L[a]	RI[b]	Developing Ovules	ng[c]	Maturing Seeds[d,e]	ng[c]	Source
a	860	(*Z*)-3-Hexenol[g]	7	(Z)-3-Hexenol[g]	13	Aldrich
b	868			**1-Hexanol**	4.8	Aldrich
c	900	Nonane	0.4			Aldrich
d	909			**Pentyl acetate**	1.2	SFU[h]
e	981	6-Methyl-5-hepten-2-one[g]	0.2	6-Methyl-5-hepten-2-one[g]	0.6	Aldrich
f	1003	(*Z*)-3-Hexenyl acetate[g]	2.4	(Z)-3-Hexenyl acetate[g]	18	SFU[i]
g	1049	*trans*-Ocimene[g]	17.8	*trans*-Ocimene[g]	10	IFF
h[f]	1099	(+)-Linalool[g]	1.8	(+)-Linalool[g]	1.4	Fluka
i	1105	Nonanal	1.6	Nonanal	1.2	Aldrich
j	1114	(*E*)-4,8-Dimethyl-1,3,7-nonatriene[g]	22.6	(*E*)-4,8-Dimethyl-1,3,7-nonatriene[g]	25	SFU[j]
k	1140			**Phenylacetonitrile**	1	Aldrich
l	1185	(*Z*)-3-Hexenyl butyrate[g]	1.8	(*Z*)-3-Hexenyl butyrate[g]	0.2	Aldrich
m	1193	Methyl salicylate[g]	1.2	Methyl salicylate[g]	2	Aldrich
n	1206	Decanal	1	Decanal	0.4	Sigma
o	1257			**2-Phenethyl acetate**	1.4	SFU[k]
p	1300	Tridecane	0.2			Aldrich
q	1308	Undecanal	0.2			SFU[l]
r	1393	*cis*-Jasmone[g]	0.5			Aldrich
s	1409	iso-Caryophyllene[g]	0.2	iso-Caryophyllene[g]	0.04	Aldrich
t	1426	*trans*-Caryophyllene[g]	5.6	*trans*-Caryophyllene[g]	0.1	Sigma
u	1449	(*E*)-β-Farnesene[g]	0.3	(*E*)-β-Farnesene[g]	0.5	Fluka
v	1490	Unknown sesquiterpene	2.8	Unknown sesquiterpene	2	
w	1505	α-Farnesene[g]	42.8	α-Farnesene[g]	94	Treatt[m]
x	1562			***trans*-Nerolidol**	5.4	Sigma
y	1600	Hexadecane	0.3			SFU[n]
z	1626	Tetradecanal	0.8			SFU[o]
aa	1715	Pentadecanal	0.2			Aldrich
bb	1926	Methyl hexadecanoate	0.5	Methyl hexadecanoate	0.4	Sigma
cc	2000	Eicosane	0.4			ICN
dd	2100	Heneicosane	0.3			Aldrich
ee	2300	Tricosane	0.2			Aldrich

[a] Letters refer to components in Fig. 3 that elicited antennal responses
[b] *RI* retention indices (Van den Dool and Kratz 1963) on a DB-5 column (see Fig. 3)
[c] Amount in nanograms (ng) present in 2 GHE [1 GHE=volatiles released from 1 g (dry weight) of ramate biomass during 1 h]; experimental dose=10 GHE
[d] All compounds in this column are constituents of the 22-component synthetic blend tested in behavioral experiments (Table 2)
[e] Bold-face compounds were present in headspace extracts only of mature samaras with maturing seeds and were constituents of the five-component synthetic blend (Table 2)
[f] At times of bioassays, the absolute configuration of linalool in headspace volatiles of boxelder trees was unknown; we anticipated and bioassayed the (-)-linalool (~85% ee) but later learned that trees emit (+)-linalool
[g] Compound that has been previously identified as released by plants in response to herbivore-induced or mechanical damage
[h] Acetylated 1-pentanol (Aldrich)
[i] Acetylated (*Z*)-3-hexen-1-ol
[j] Synthesized by Grigori Khaskin in Gries-laboratory
[k] Acetylated 2-phenylethanol (Fluka)
[l] Oxidized 1-undecanol
[m] Treatt, USA Inc. (Lakeland, Florida, 33805-7637)
[n] Standard
[o] Oxidized 1-tetradecanol (Aldrich)

retention indices (Van den Dool and Kratz 1963) and mass spectra with those reported in the literature [(*Z*)-3-hexen-1-ol, 1-hexanol, nonane, pentyl acetate, 6-methyl-5-hepten-2-one, *trans*-ocimene, linalool (enantiomeric composition not known), nonanal, methyl salicylate, decanal, 2-phenethyl acetate, tridecane, *cis*-jasmone, (*E*)-β-farnesene, α-farnesene, *trans*-nerolidol, hexadecane, eicosane, heneicosane, tricosane (Adams 1989); (*E*)-4,8-dimethyl-1,3,7-nonatriene, phenylacetonitrile, *trans*-caryophyllene, tetradecanal, pentadecanal, methyl hexadecanoate (McLafferty 1994-98); (*Z*)-3-hexenyl acetate, (*Z*)-3-hexenyl butyrate, undecanal (Jennings and Shibamoto 1980); *iso*-caryophyllene (Joulain and König 1998)] and with those of authentic standards.

General Bioassay Procedures Walking responses of 3-day-starved adults or 3- to 4-day-starved fifth instar nymphs to 10 GHE of headspace extracts (a dose found suitable in preliminary experiments) or to synthetic chemicals were bioassayed at 25–28°C in a horizontal Y-shaped Pyrex® glass olfactometer (stem 20×2.5 cm ID; side-arms at 120°; 18 cm long) illuminated from above by two horizontal fluorescent mercury lamps (GE™, Ecolux; 5,000 K, 32 W). For each replicate, treatment and control stimuli were randomly (coin toss) assigned to and pipetted onto Whatman® 1.5-cm filter paper, positioned near the orifices of the side-arms. In all experiments, a water aspirator drew humidified air at 0.5 l min^{-1} through the Y-tube, carrying volatiles from the treated filter paper to the insect released from a holding tube at the base of the Y-tube. For each replicate, a clean Y-tube and a new insect were employed. An insect was scored as a responder if it approached within 2.5 cm of a stimulus within 5 min. Nonresponding insects were excluded from statistical analyses. Olfactometers were washed in warm water with Sparklene™ detergent, rinsed with cold tap and distilled water, and oven-dried at ca. 125°C for at least 1 h.

Specific Experiments Experiments 1–8 were designed to test whether or not the attractiveness of pistillate and staminate trees changes as the growing season progresses (Fig. 1; Table 2). Experiments 9–14 were designed to identify semiochemicals mediating attraction of BEBs to volatiles of pistillate trees with mature samaras by testing two synthetic blends: one consisting of the 22 chemicals that elicited an antennal response (Table 1) and the other

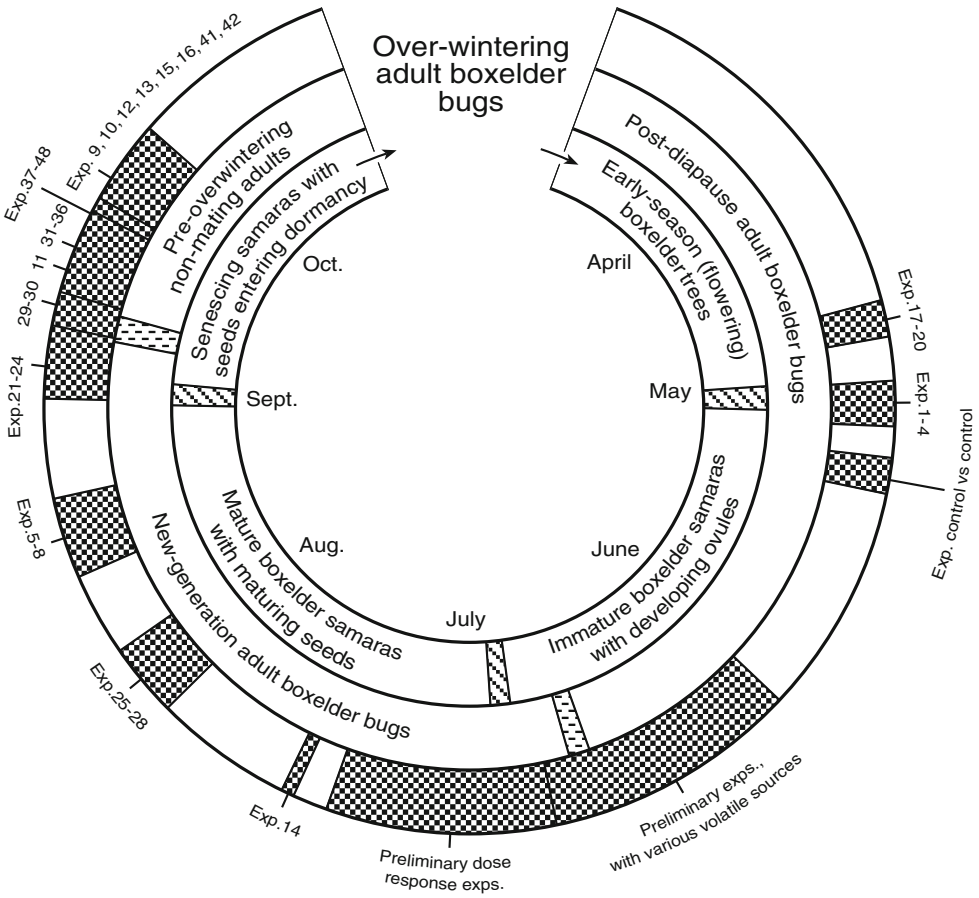

Fig. 1 Illustration depicting aspects of the seasonal phenology of boxelder trees, *A. negundo*, and western boxelder bugs, *B. rubrolineata*, and the time of season (*shaded sections in the outer ring*) during which experiments were conducted

Table 2 Stimuli and experimental insects tested in Y-tube olfactometer experiments 1–42

Exp.[a,b,c]	Treatment stimuli[d,e]	Bioassay insects	N[f]
1	Volatiles of staminate trees in early spring[g]	Post-diapause ♀[o]	49
2	Volatiles of staminate trees in early spring[g]	Post-diapause ♂[o]	52
3	Volatiles of pistillate trees in early spring	Post-diapause ♀[o]	50
4	Volatiles of pistillate trees in early spring	Post-diapause ♂[o]	50
5	Volatiles of pistillate trees with immature samaras[h]	New-generation ♀[p]	27
6	Volatiles of pistillate trees with immature samaras[h]	New-generation ♂[p]	27
7	Volatiles of pistillate trees with mature samaras[i]	New-generation ♀[p]	27
8	Volatiles of pistillate trees with mature samaras[i]	New-generation ♂[p]	27
9	5-Component synthetic blend[j]	Pre-overwintering ♀[p]	32
10	5-Component synthetic blend[j]	Pre-overwintering ♂[p]	35
11	5-Component synthetic blend[j]	5th-Instar nymphs	26
12	22-Component synthetic blend[k]	Pre-overwintering ♀[p]	32
13	22-Component synthetic blend[k]	Pre-overwintering ♂[p]	24
14	22-Component synthetic blend[k]	5th-instar nymphs	29
15	Volatiles of pistillate trees with senescent samaras[l]	Pre-overwintering ♀[p]	29
16	Volatiles of pistillate trees with senescent samaras[l]	Pre-overwintering ♂[p]	23
17	5-Component synthetic blend[j]	Post-diapause ♀[o]	25
18	5-Component synthetic blend[j]	Post-diapause ♂[o]	24
19	Volatiles of staminate trees in early spring[g]	Post-diapause ♀[o]	25
20	Volatiles of staminate trees in early spring[g]	Post-diapause ♂[o]	25
21	5-Component synthetic blend[j]	New-generation ♀[q]	21
22	5- Component synthetic blend[j]	New-generation ♂[q]	20
23	Volatiles of staminate trees in early spring[g]	New-generation ♀[q]	21
24	Volatiles of staminate trees in early spring[g]	New-generation ♂[q]	25
25	5-Component synthetic blend[j]	Immature ♀[q]	23
26	5-Component synthetic blend[j]	Immature ♂[q]	22
27	5-Component synthetic blend[j]	Reproductive ♀[r]	25
28	5-Component synthetic blend[j]	Reproductive ♂[r]	25
29	Phenylacetonitrile + 2-phenethyl acetate (5 + 7 ng)[m]	New-generation ♀[q]	30
30	Phenylacetonitrile + 2-phenethyl acetate (5 + 7 ng)[m]	New-generation ♂[q]	32
31	5-Component synthetic blend[j]	New-generation ♀[q]	20
32	5-Component synthetic blend[j]	New-generation ♂[q]	20
33	Phenylacetonitrile + 2-phenethyl acetate (5 + 7 ng)[m]	New-generation ♀[q]	20
34	Phenylacetonitrile + 2-phenethyl acetate (5 + 7 ng)[m]	New-generation ♂[q]	20
35	2-Phenethyl acetate (10 ng)	New-generation ♀[q]	18
36	2-Phenethyl acetate (10 ng)	New-generation ♂[q]	18
37	2-Phenethyl acetate (10 ng)	Pre-overwintering ♀[q]	20
38	Phenylacetonitrile (10 ng)[n]	Pre-overwintering ♀[q]	20
39	5-Component synthetic blend[j]	Pre-overwintering ♀[q]	20
40	Phenylacetonitrile (10 ng)[n]	Pre-overwintering ♂[q]	22
41	Phenylacetonitrile (10 ng)[n]	5th-Instar nymphs	20
42	2-Phenethyl acetate (10 ng)	5th-Instar nymphs	20

[a] Experiments 1-4, 5-8, 9-16, 17-20, 21-24, 25-28, 29-30, 31-34, 35-36, 37-40, and 41-42, respectively, were run in parallel
[b] Dose–response experiments (not listed here) determined that 10 GHE was a suitable dose
[c] A control- vs. control-stimulus experiment revealed no side bias of the bioassay setup
[d] The control stimulus (pentane) consisted of the same amount of solvent (10 µl) as dispensed with the treatment stimulus
[e] Headspace chemicals of early-season staminate and pistillate trees in 2006 were tested in parallel at 10 GHE, at quantities of ca. 900 ng and ca. 1,000 ng, respectively. In 2007, volatiles of early-season staminate trees, when tested in parallel to either the five- or 22-component synthetic blends, were tested at ca. 930 ng in 10-µl aliquots (equivalent to the amount of the 22 antennal stimulatory components in pistillate trees with maturing seeds at 10 GHE)
[f] N=number of insects bioassayed
[g] Bearing pollen
[h] Immature samaras contain ovules
[i] Mature samaras contain maturing seeds
[j] The blend comprised 1-hexanol (24 ng), pentyl acetate (6 ng), phenylacetonitrile (5 ng), 2-phenethyl acetate (7 ng), and *trans*-nerolidol (27 ng)
[k] For blend constituents and their quantities, see columns 5 and 6 of Table 1
[l] Senescent samaras contain ripening seeds entering dormancy
[m] The amounts of synthetic test chemicals reflect those of natural constituents in head space volatiles of pistillate trees with maturing seeds at 10 GHE
[n] The amount of synthetic test chemical reflects that of the natural constituent in head space volatiles of early-season staminate trees at 10 GHE
[o] Adult age unknown
[p] Less than 14-days-old adults (note: pre-overwintering adults are in reproductive diapause)
[q] Four- to 6-day-old adults (note: pre-overwintering adults will enter reproductive diapause)
[r] More than 30-days-old adults

consisting of five components (1-hexanol, pentyl acetate, phenylacetonitrile, 2-phenethyl acetate, and *trans*-nerolidol) that were selected based on their changing seasonal abundance. Experiments 15 and 16 were "negative controls" that used volatiles from pistillate trees with senescing foliage and samaras. Experiments 17–28 were designed to explore whether the attractiveness of volatile blends, from previous experiments, varied with time of season and the insect's life history stage and reproductive status. Experiments 29–42 aimed to determine the essential components (s) in the five-component blend. Experiments 29 and 30 tested a two-component blend of phenylacetonitrile and 2-phenethyl acetate. Follow-up experiments 31–34 tested this two-component blend against the five-component blend, while experiments 35–42 tested single components against the different sexes and life stages.

Statistical Analyses Data of all experiments were analyzed by the χ^2 goodness-of-fit test, using Yates' correction for continuity (α=0.05; Zar 1999). Logistic regression analyses (Table 3) were used to test for effects of insect sex, developmental stage, and reproductive state, host type, season, and synthetic chemical blend, and interactions thereof (α=0.05). Fisher's exact test was used to test for differences in response by fifth-instar nymphs to synthetic chemical blends (α=0.05). All analyses employed JMP™ software (SAS®, Cary, NC, USA).

Results

Headspace volatiles of early-season staminate, but not of pistillate, trees attracted both male and female post-diapause BEBs (Fig. 2; experiments 1–4). Host type had a significant effect on the insects' responses, but sex of insect did not (Table 3). Volatiles of branches with immature samaras did not attract females or males (Fig. 2; experiments 5–6), whereas those of branches with mature samaras did (Fig. 2; experiments 7–8). Host season had a significant effect on the insects' responses, but sex of insect did not (Table 3).

In GC-EAD recordings of extracts that were found attractive in experiments 7 and 8, 22 volatile components (Table 1) elicited antennal responses, most of which are shown in Fig. 3. We considered likely essential components of an attractive blend to be those that elicited an antennal response (Fig. 3) and varied in relative abundance in volatile profiles of pistillate trees throughout the season (Fig. 4). Based on these criteria, five components [1-hexanol, pentyl acetate, phenylacetonitrile, 2-phenethyl acetate, and *trans*-nerolidol] were selected and bioassayed at quantities and ratios equivalent to those found in 10 GHE of pistillate trees with mature samaras (Table 1). In experiments 9–14 (Fig. 5), both the five-component and the 22-component blends of antennal stimulatory components (Fig. 3; Table 1) attracted females (Fig. 5, experiments 9, 12), males (Fig. 5, experiments 10, 13), and fifth-instar nymphs (Fig. 5, experiments 11, 14). These results suggested that essential semiochemicals were present in the five-component blend. In experiments 9–14, insect sex and developmental stage had no effect on the insects' responses (Table 3).

Considering that these five components were present at much reduced quantities in headspace volatile extracts of trees with senescing samaras (Fig. 4), we predicted that such extracts would not be attractive to BEBs. The results of experiments 15 and 16 confirmed this prediction (Fig. 5). Thus, host season had a significant effect on the insects' responses but sex of insect did not (Table 3).

The insects' responses to attractive blends did not vary with time of season, the insects' life history stage, or sex (Fig. 6, experiments 17–24; Table 3), but it did vary with the insects' reproductive state (Fig. 6, experiments 25–28; Table 3). In particular, reproductively active males, but not females (Fig. 6, experiment 27, 28), were attracted to the five-component blend. The attraction of post-diapause and new-generation females and males to the volatile extract of early-season staminate trees (Fig. 6, experiments 19, 20, 23, 24) suggested that it contained essential constituents. The presence of phenylacetonitrile and 2-phenethyl acetate (trace) in this extract was confirmed by GC-MS analysis.

In experiments 29 and 30, the synthetic blend of phenylacetonitrile and 2-phenethyl acetate attracted females and males (Fig. 7). In follow-up and parallel-run experiments 31–34, this 2-component blend was as effective as the five-component blend in attracting females and males (Fig. 7; Table 3). In experiments 35 and 36, 2-phenethyl acetate attracted both females and males (Fig. 7). In parallel-run experiments 37–39, 2-phenethyl acetate and phenylacetonitrile as single components were as effective as the five-component blend in attracting females (Table 3). Similarly, in experiment 40, phenylacetonitrile attracted males. In experiments 29–40, neither sex nor blend composition affected the insects' responses (Table 3). In parallel-run experiments 41–42, phenylacetonitrile, but not 2-phenethyl acetate, attracted fifth-instar nymphs (Fig. 7).

Discussion

Our data support the hypotheses that (1) BEBs are attracted to volatiles of early-season staminate trees bearing pollen; (2) volatile profiles of pistillate trees change as the season progresses; (3) pistillate trees with mature samaras are

Table 3 Logistic regression analyses of the effects of sex, developmental stage, and reproductive state of *B. rubrolineata*, as well as host season, host type, synthetic chemical blends, or interactions (×) between them, tested in experiments 1–41

Experiments	Sources	Effects	*df*	χ^2	*P* value
1–4	Early-season	Insect sex	1	0.163	NS
	Staminate tree	Host type	1	4.64	**0.03***
	Pistillate tree	Sex × type	1	0.02	NS
5–8, 15–16	Immature ovules	Insect sex	1	0.112	NS
	Maturing seeds	Host season	2	6.965	**0.03***
	Senescing tree	Sex × season	2	0.147	NS
5–8	Immature ovules	Insect sex	1	0.004	NS
	Maturing seeds	Host season	1	5.032	**0.02***
		Sex × season	1	0.004	NS
5–6, 15–16	Immature ovules	Insect sex	1	0.131	NS
	Senescing tree	Host season	1	0.004	NS
		Sex × season	1	0.131	NS
7-8, 15–16	Maturing seeds	Insect sex	1	0.163	NS
	Senescing tree	Host season	1	5.593	**0.02***
		Sex × season	1	0.074	NS
9–10, 15–16	5-SB[a]	Insect sex	1	0.342	NS
	Senescing trees	Season	1	4.319	**0.04***
		Sex × season	1	0.031	NS
9–14	5-SB	Insect sex[d]	2	2.914	NS
	22-SB[b]	Synthetic blends	1	0.24	NS
		Sex × blends	2	0.467	NS
		Insect stage[e]	1	3.003	NS
		Stage × blend	1	0.134	NS
17–24	5-SB	Insect sex	1	0.012	NS
	Early-season	Insect stage[f]	1	0.001	NS
	Staminate tree	Sex × stage	1	0.338	NS
		Host blend	1	1.396	NS
		Stage × blend	1	0.004	NS
25–28	5-SB	Insect sex	1	1.499	NS
		Reproductive state	1	7.179	**0.007****
		Sex × state	1	0.251	NS
25, 27	5-SB	Reproductive state	1	6.335	**0.01****
26, 28	5-SB	Reproductive state	1	1.927	NS
29–30	2-SB[c]	Insect sex	1	0.158	NS
31–34	2-SB	Insect sex	1	1.700	NS
	5-SB	Synthetic blend	1	0.581	NS
		Sex × blend	1	0.014	NS
37–39	5-SB	Synthetic blend	2	0.784	NS
	PAN				
	2-PEOAc				
31–32, 35–36, 38, 40	2-SB	Insect sex	1	0.35	NS
	PAN	Host blend	2	0.151	NS
	2-PEOAc	Sex × blend	2	0.293	NS
38, 40, 41	PAN	Insect sex	2	0.105	NS

NS not significant, *PAN* phenylacetonitrile, *2-PEOAc* 2-phenethyl acetate

[a] *5-SB* Synthetic blend of the 5 antennally active volatiles of pistillate trees with maturing seeds, not present in trees with developing ovules (Table 1; Fig. 3)

[b] *22-SB* Synthetic blend of all 22 antennal-active volatiles of pistillate trees with maturing seeds (Table 1; Fig. 3)

[c] *2-SB* Synthetic blend of phenylacetonitrile and 2-phenethyl acetate

[d] Insect sex: female, male, or fifth-instar nymph

[e] Insect developmental stage: adults or fifth-instar nymphs

[f] Insect stage: post-diapause adults or new-generation adults

*$P \leq 0.05$, **$P \leq 0.01$; significant effect (L-R χ^2 test)

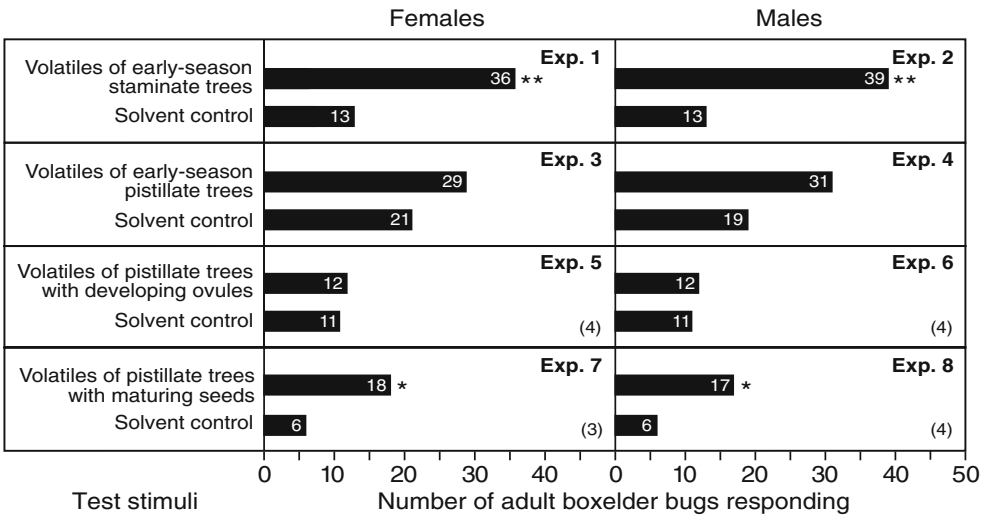

Fig. 2 Responses of post-diapause adult female and male western boxelder bugs, *B. rubrolineata*, in experiments 1–4 and of new-generation virgin adult females and males in experiments 5–8, to headspace volatile extracts of staminate (♂) and pistillate (♀) host trees, *A. negundo*, in Y-tube olfactometers. *Numbers in parentheses* are nonresponding insects. An *asterisk* indicates a significant preference for a particular test stimulus (χ^2 test; $^*P<0.05$; $^{**}P<0.01$)

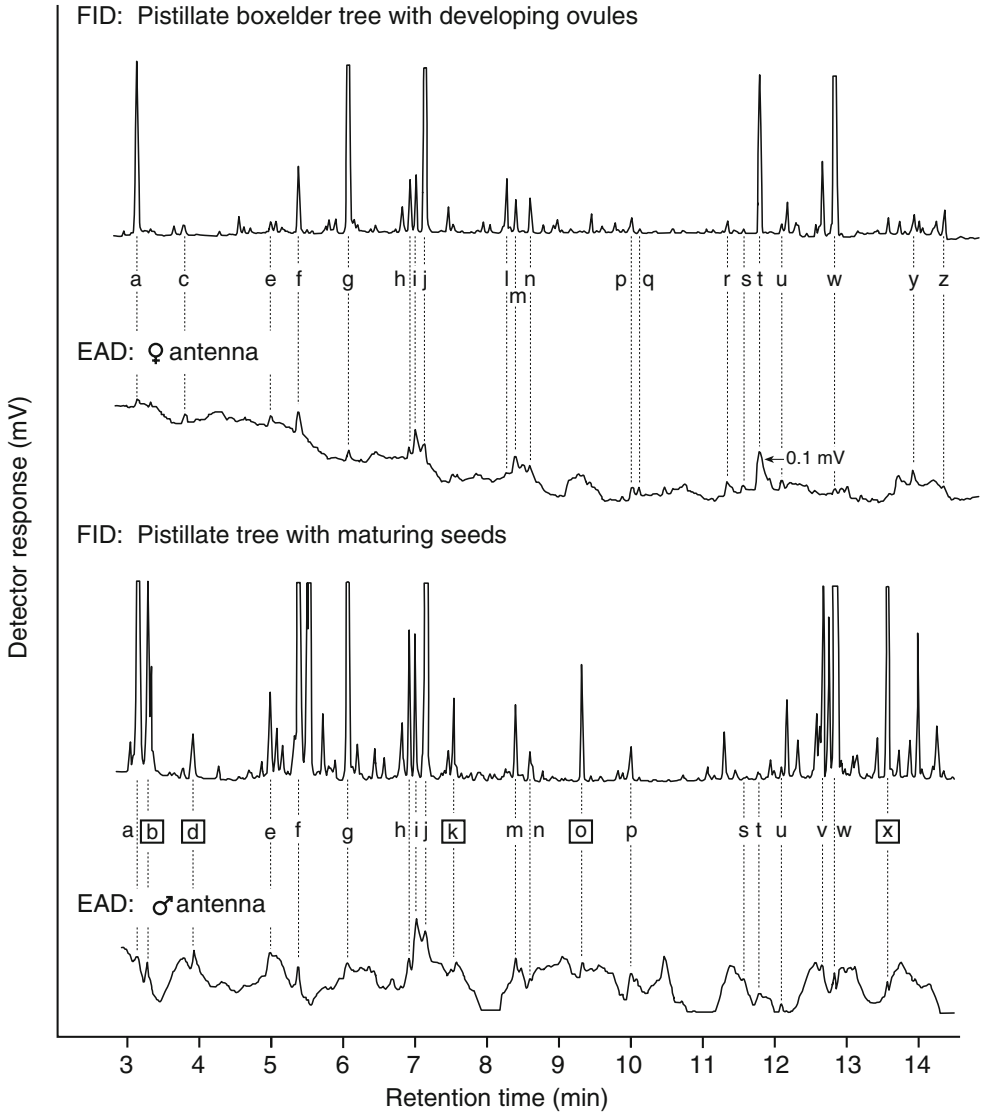

Fig. 3 Representative recordings ($N=3$) of flame ionization detector (*FID*) and electroantennographic detector (*EAD*: female or male *B. rubrolineata* antenna) responses to aliquots of headspace extract from pistillate (♀) trees with immature samaras (*top*) and mature samaras (*bottom*). Compounds *b*, *d*, *k*, *o*, and *x*, respectively, are 1-hexanol, pentyl acetate, phenylacetonitrile, 2-phenethyl acetate, and *trans*-nerolidol. Additional antennal stimulatory components are listed in Table 1. Chromatography: DB-5 column; splitless injection; temperature of injection port and FID: 240°C; temperature program: 50°C (2 min), 10°C min^{-1} to 280°C (5 min)

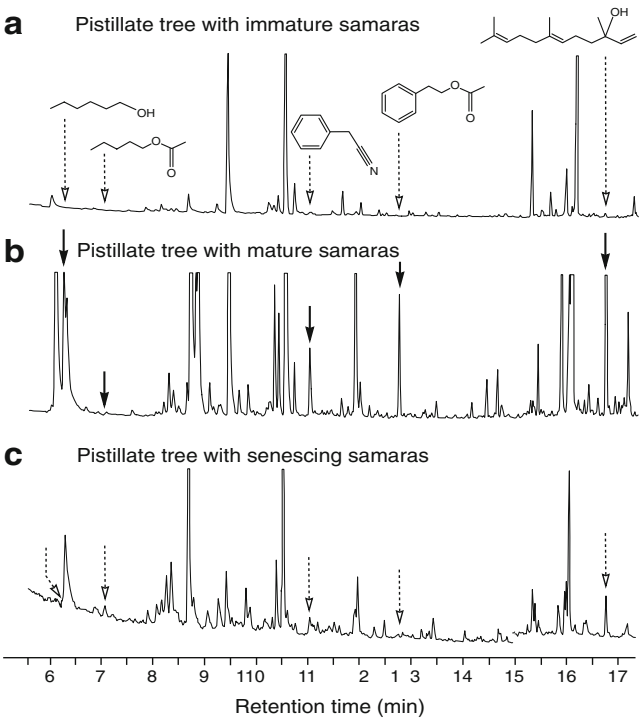

Fig. 4 Headspace volatiles of pistillate boxelder trees, *A. negundo*, with immature samaras (**a**), mature samaras (**b**), or senescing samaras (**c**). Note the absence of 1-hexanol, pentyl acetate, phenylacetonitrile, 2-phenethyl acetate, and *trans*-nerolidol in **a**, their prevalence in **b**, and decline in **c**. Chromatography: as in Fig. 3 except FID: 250°C

attractive to BEBs; and (4) specific semiochemicals mediate attraction of BEBs to boxelder trees with pollen or seed resources.

Insects emerging from overwintering diapause seek food sources that increase their reproductive potential prior to mating (Tanaka et al. 1987; Tanaka and Wolda 1988; Kalberer et al. 2001). Post-diapause female and male BEBs are attracted to volatile blends of early-season pollen-bearing staminate boxelder trees, but also feed on new-growth tissue of both staminate and pistillate trees. Pollen is a rich supply of protein for the growth, development, and performance of many phytophagous insects (Strong et al. 1984) and likely supplements the diet of BEBs, which are also facultative cannibals and insect scavengers (Brown and Norris 2004). Similarly, western conifer seed bugs, *Leptoglossus occidentalis*, and plum curculio beetles, *Conotrachelus nunuphar*, feed on staminate flowers in the spring (Blatt 1997; Leskey and Prokopy 2000). BEBs may feed on pistillate trees in the spring because they have fed on seeds the preceding year and then over-wintered nearby.

Locating host trees rapidly is an effective means of exploiting ephemeral food resources and explains why both *Jadera* spp. (Aldrich et al. 1990a) and BEBs respond to semiochemicals that indicate seed availability (Schwarz 2008). As suggested for the respective hosts of the heteropteran seed feeders *Jadera* (Carroll and Loye 1987) and *Pachycoris* (Grimm and Führer 1998), boxelder trees may escape seed predation by producing mature seeds late in the season with little time for BEB populations to increase and significant seed predation to take place.

Although volatile blends of early- and late-season pistillate trees were not attractive in bioassays, field observations indicate that pistillate trees in spring and early summer suffer feeding damage by BEBs and other herbivores, such as aphids and thrips. Clipped branches of

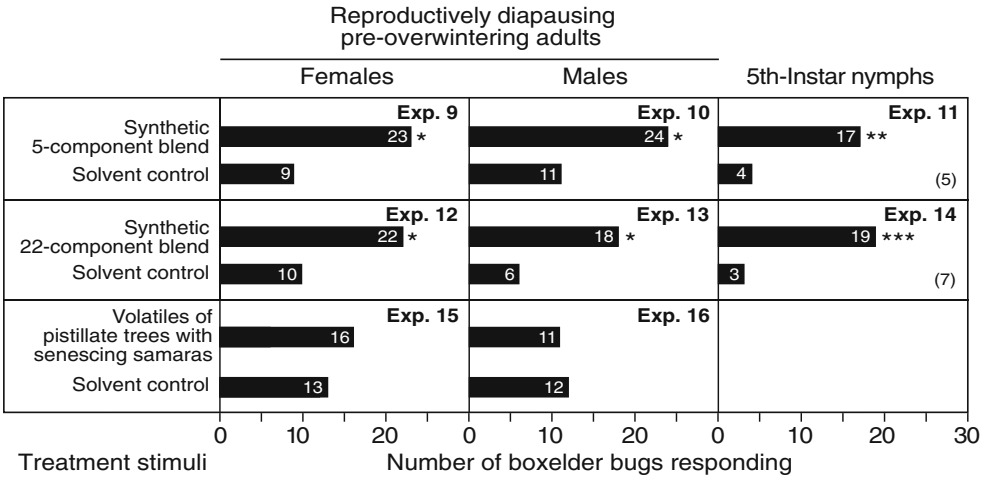

Fig. 5 Responses of fifth-instar nymph and virgin adult female and male western boxelder bugs, *B. rubrolineata*, in Y-tube olfactometer experiments to five- or 22-component synthetic blends of candidate semiochemicals and to headspace volatile extract of pistillate (♀) trees with senescing samaras. Constituents of the synthetic blends are listed in Table 1. *Numbers in parentheses* indicate numbers of nonresponding insects. An asterisk indicates a significant preference for a particular test stimulus (χ^2 test; *$P<0.05$, **$P<0.01$, ***$P<0.001$)

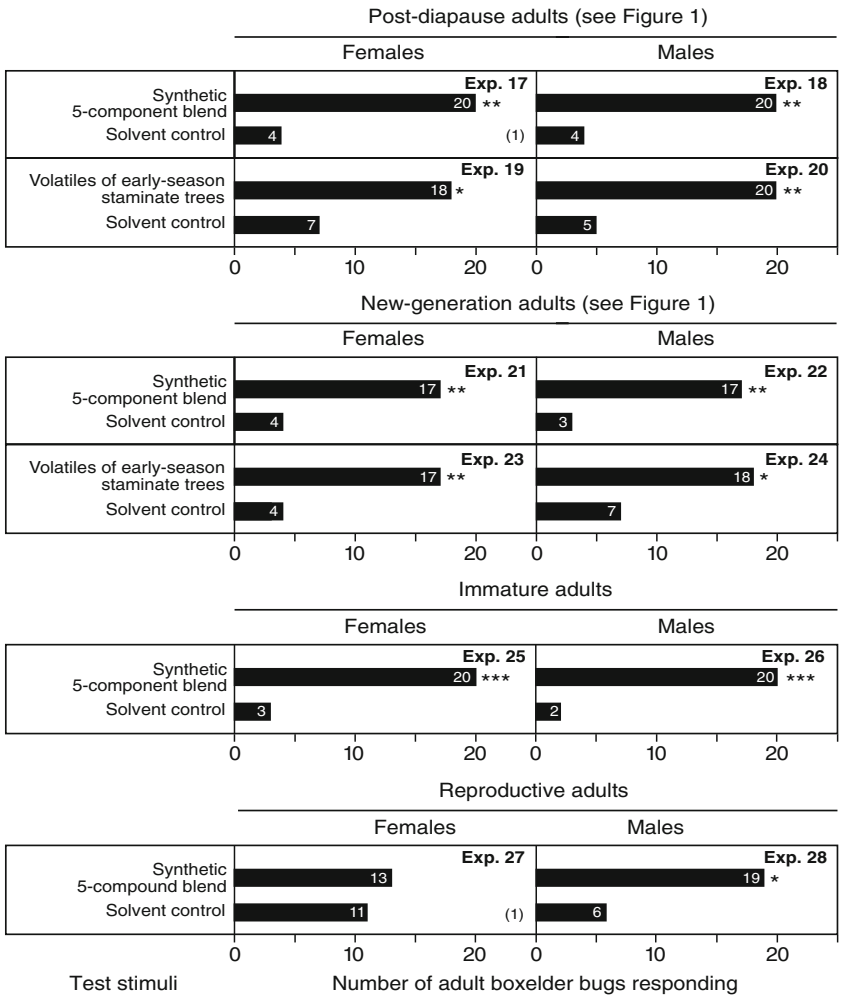

Fig. 6 Responses of female and male western boxelder bugs, *B. rubrolineata*, of different life history stage and reproductive status to a five-component synthetic blend (see Table 1) or to headspace volatile extract of early-season staminate (♂) trees. *Numbers in parentheses* indicate numbers of nonresponding insects. An *asterisk* indicates a significant preference for a particular test stimulus (χ^2 test; *$P<0.05$, **$P<0.01$, ***$P<0.001$)

pistillate trees, with BEBs feeding or with mechanical damage, were more attractive to BEBs, and they released higher levels of phenylacetonitrile and 2-phenethyl acetate, than did clipped branches with foliage devoid of insect or mechanical damage (data not shown). This suggests that herbivory may promote the apparency of trees with seeds near maturity, thus influencing colonization by BEBs. Many of the compounds that elicited antennal responses from BEBs have been identified from plants in response to herbivore or mechanical damage and may be used as foraging cues by herbivores (Walling 2000 and references therein).

Availability and quality of food resources affect the decisions of insects regarding when to stay and feed and when to leave (Dingle 1974). When boxelder seeds enter dormancy and BEBs cease to reproduce, phenylacetonitrile and 2-phenethyl acetate levels released by the plant have declined to trace quantities, rendering the volatile blend unattractive to BEBs, even though in laboratory bioassays pre-overwintering BEBs are attracted to synthetic blends that contain phenylacetonitrile and 2-phenethyl acetate. Pre-overwintering BEBs that feed on senescing hosts (JJS, personal observation) are probably not attracted to them but have remained to feed on "last-minute" nutrients that may be needed to complete development as adults as well as to build nutritional reserves for overwintering survival. This has been observed previously in ladybird beetles, *Coccinella septempunctata* (Barron and Wilson 1998).

The reproductive state of BEBs modulates their responses to host semiochemicals. Gravid females no longer responded to the five-component blend, suggesting a physiological change in accord with the insect's ecology. Gravid females likely are already present on resources needed for food and oviposition and therefore may no longer engage in host-finding behavior. Moreover, ovipositing females may trade off the mobility required in host-finding for increased fecundity. In female cotton stainer seed bugs, *Dysdercus* spp., availability of food (and oviposition sites) induced histolysis of wing muscles and

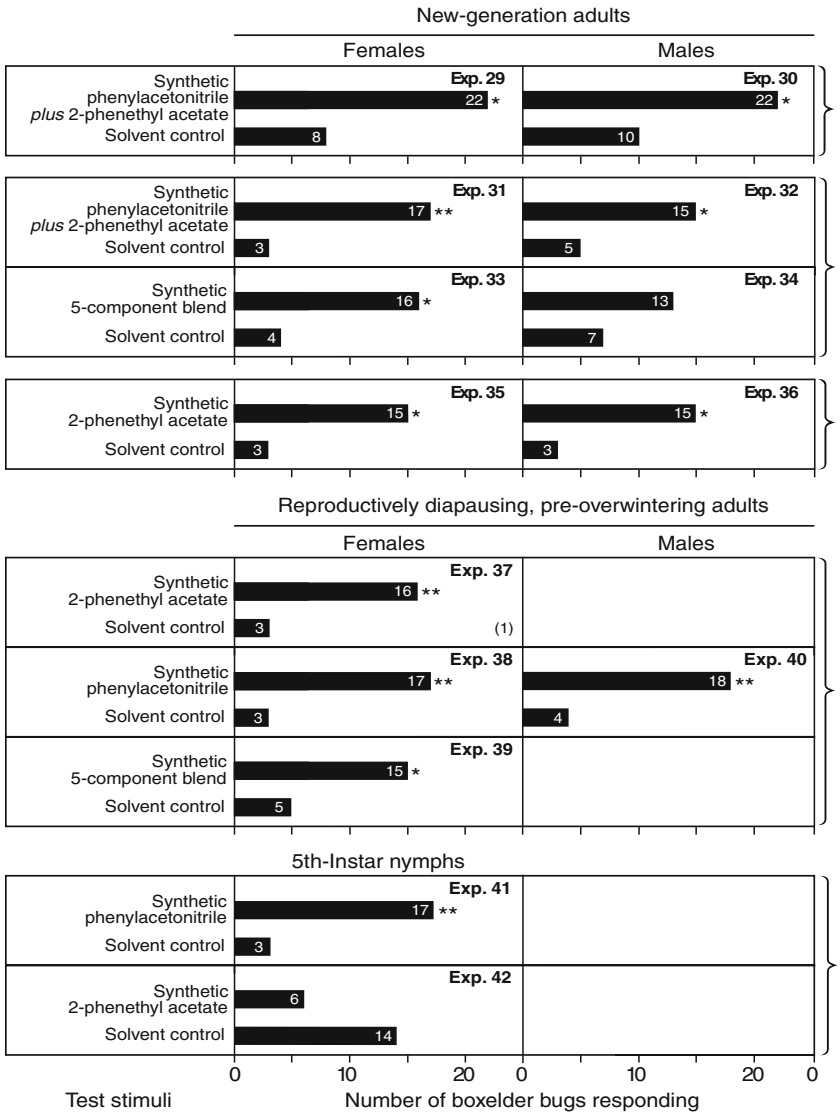

Fig. 7 Responses of fifth-instar nymph and sexually immature adult female and male western boxelder bugs, *B. rubrolineata*, to various synthetic blends of candidate semiochemicals. Constituents of the five-component blend are listed in Table 1. *Numbers in parentheses* indicate numbers of nonresponders. An asterisk indicates a significant preference for a particular test stimulus (χ^2 test; *$P<0.05$, **$P<0.01$). Note: experiment 40 was run not in parallel with experiments 37–39; in experiments 41 and 42, the test stimulus had a significant effect on response of fifth-instar nymphs (Fisher's Exact Test, $\alpha=0.05$, one-tailed, $df=1$, $P<0.001$)

rapid development of eggs (Dingle and Arora 1973). That reproductively active BEB males, unlike sedentary gravid females, remain responsive to the five-component blend may allow them to seek further food resources and mating opportunities with females.

Phenylacetonitrile appears to be an essential host tree kairomone for BEBs as it was the only single compound that attracted both adults and nymphs. Plants that produce phenylacetonitrile are known to store secondary metabolites (e.g., flavonoids, cyanogenic glycosides, glucosinolates) that specialist herbivores may sequester for aposematic defense. BEBs attain their aposematic coloration by ingesting flavonoids (Palmer and Knight 1924) and may gain secondary defense by ingesting or metabolizing cyanogenic toxins from seeds of host trees, as do other aposematic true bugs (Braekman et al. 1982; Aldrich et al. 1990a). Phenylacetonitrile has been reported as a semiochemical metabolite of benzylglucosinolates of oil seed rape, *Brassica napus* and *Brassica rapa*, and attracts both the specialist cabbage seed weevil, *Ceutorhynchus assimilis* (Bartelt et al. 1997; Smart and Blight 1997), and the specialist cabbage butterfly, *Pieris rapae* (Ômura et al. 1999). 2-Phenethyl acetate is a widespread constituent of floral and fruit scents (Knudsen and Tollsten 1993) that likely forms from 2-phenylethanol by alcohol acetyltransferases unique to plants (Guterman et al. 2006).

In summary, our data indicate that specific semiochemicals from the boxelder tree attract the specialist boxelder bug. Phenylacetonitrile attracts BEBs to early-season, pollen-bearing staminate trees and to midseason pistillate trees with maturing seeds. By responding to phenylacetonitrile, BEBs appear to track and exploit nutrient-rich food sources.

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RAPID COMMUNICATION

Lichen Substances Prevent Lichens from Nutrient Deficiency

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Abstract The dibenzofuran usnic acid, a widespread cortical secondary metabolite produced by lichen-forming fungi, was shown to promote the intracellular uptake of Cu^{2+} in two epiphytic lichens, *Evernia mesomorpha* and *Ramalina menziesii*, from acidic, nutrient-poor bark. Higher Cu^{2+} uptake in the former, which produces the depside divaricatic acid in addition to usnic acid, suggests that this depside promotes Cu^{2+} uptake. Since Cu^{2+} is one of the rarest micronutrients, promotion of Cu^{2+} uptake by lichen substances may be crucial for the studied lichens to survive in their nutrient-poor habitats. In contrast, study of the uptake of other metals in *E. mesomorpha* revealed that the intracellular uptake of Mn^{2+}, which regularly exceeds potentially toxic concentrations in leachates of acidic tree bark, was partially inhibited by the lichen substances produced by this species. Inhibition of Mn^{2+} uptake by lichen substances previously has been demonstrated in lichens. The uptake of Fe^{2+}, Fe^{3+}, Mg^{2+}, and Zn^{2+}, which fail to reach toxic concentrations in acidic bark at unpolluted sites, although they are more common than Cu^{2+}, was not affected by lichen substances of *E. mesomorpha*.

Keywords Lichenized Ascomycetes · Dibenzofurans · Depsides · Metal homeostasis · Copper

Introduction

Lichens are known for their ability to cope with extreme environments. They can adapt to extreme temperatures, drought, inundation, salinity, high concentrations of heavy metals (Nash 2008), or even survive in outer space (Sancho et al. 2007). Another outstanding character of lichens is tolerance to nutrient-poor environments. Mechanisms that enable a lichen to deal with shortages of nutrients have been poorly scrutinized, although most cannot compete with vascular plants at well-supplied sites.

Lichen substances are a chemically diverse group of more than 800 mostly phenolic compounds largely specific to lichen-forming fungi. They recently were shown to specifically inhibit the intracellular uptake of toxic amounts of transition metals, whereas other metals absent at toxic concentrations from the environment of the lichen could pass (Hauck 2008). Due to the high specificity of the role of lichen substances in metal tolerance, we tested the hypothesis that the opposite effect, the promotion of the uptake of metals needed as micronutrients, may be realized in the lichen symbiosis in nutrient-poor environments. We studied Cu^{2+} uptake in lichens with usnic acid (UA), a widespread cortical dibenzofuran in lichen-forming fungi. This example was selected, as Cu^{2+} is, after Mo, usually the rarest micronutrient in the microhabitats of epiphytes of nutrient-poor bark (Gauslaa 1995). Moreover, UA forms complexes with Cu^{2+} (Takani et al. 2002). We selected two UA-producing epiphytic lichens characteristic of nutrient-poor, acidic bark, *Evernia mesomorpha* Nyl. and *Ramalina menziesii* Taylor. The former lichen produces the depside divaricatic acid (DA) in addition to UA, whereas the latter lichen only synthesizes UA.

Methods and Materials

Lichens were collected in Mongolia (Khentey, Eroo, Khonin Nuga; *E. mesomorpha*) and the USA (Oregon, Benton County, Porter Lake; *R. thrausta*). Thalli were

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stored in air-dry condition at −18°C in the dark before the experiment. After thawing, extracellular lichen substances were extracted with acetone from one half of the lichen thalli. Samples were submersed in acetone (4×10 min). The efficacy of this treatment at removing lichen substances was controlled by high-pressure liquid chromatography by using a reverse-phased column and gradient elution. Viability of lichen thalli was controlled by measuring the chlorophyll fluorescence yield (Φ_2) of light-adapted samples at photosystem II after the acetone treatment with a PAM-2100 chlorophyll fluorometer (Walz, Effeltrich, Germany).

Five pieces of the fruticose thalli of up to 5-cm length were used in each replicate sample and combined on a Petri dish with a moist cellulose filter. For acclimatization, Petri dishes with thalli were stored in a growth chamber for 2 days at 80% RH, a day temperature (for 13 h daily) of 13°C with a photon flux density of 30 μmol m^{-2} s^{-1}, and a night temperature of 10°C. Lichens then were exposed for 30 min to 50 ml of 20 μM $CuCl_2$, which is typical of the microhabitats of the studied lichens under unpolluted conditions on a shaker at pH 4. The uptake of Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} from chloride salt solutions was studied in *E. mesomorpha* in comparison. These salts were applied at a higher concentration (100 μM) than $CuCl_2$ because their concentrations in stemflow, throughfall, and bark, which form important nutrient sources for epiphytes, are higher than that of Cu^{2+} (Gauslaa 1995). Moreover, these metals cause less membrane damage than Cu^{2+}, which would impair the experiment. After exposure to the metal solution, the extra- and intracellular cations were sequentially extracted. For this purpose, samples were shaken with deionized water (2×20 ml) to remove free apoplastic ions. Metal ions bound to hydroxylic or carboxylic exchange sites of the cell wall were exchanged by shaking samples with $NiCl_2$ (2×20 ml). Afterwards, all samples were shaken with acetone (2×20 ml) to remove metal ions potentially bound to extracellular lichen substances. Samples were dried at 105°C, homogenized, and digested with 65% HNO_3 in order to analyze intracellular concentrations of Cu, Fe, Mg, Mn, or Zn with atomic absorption spectrometry (AAS Vario 6, Analytik Jena, Germany). The measuring error inherent to the AAS amounted to <1%.

Statistical analyses were computed with SAS 6.04 software (SAS Institute Inc., Cary, NC, USA). All data are given as arithmetic means±standard error and were tested for normal distribution with the Shapiro–Wilk test. Samples were tested for significant differences with Student's *t* test for pairwise comparisons.

Results and Discussion

Samples containing their natural content of lichen substances took up significantly more Cu^{2+} than acetone-extracted samples, both in *E. mesomorpha* (*t* test, $P \leq 0.01$, $df=4$) and in *R. menziesii* ($P \leq 0.05$; Fig. 1a, b). The promotion of metal uptake was highly specific for Cu^{2+}. Uptake of Fe^{2+}, Fe^{3+}, Mg^{2+}, and Zn^{2+} in *E. mesomorpha* was not influenced by lichen substances. Intracellular uptake of Mn^{2+} was even reduced ($P \leq 0.05$; Fig. 1c). This makes sense, as peak concentrations of Mn^{2+} leached from the canopy are known to limit the abundance of lichens on acidic bark (Hauck and Paul 2005), as has been found in conifers and some broad-leaved trees such as beech and oak. Hence, reduced uptake broadens the ecological niche of lichens, although Mn^{2+} is essential in minor amounts. Partial inhibition of Mn^{2+} uptake by lichen substances is already known from epiphytic lichens at Mn^{2+}-rich sites (Hauck 2008).

Stronger promotion of Cu^{2+} uptake in *E. mesomorpha* than in *R. menziesii* (Fig. 1) suggests that DA in addition to UA could contribute to Cu^{2+} uptake. However, although members of the same family (Parmeliaceae), both lichens differ in their morphology, which is also likely to affect element uptake. Complex formation of UA and Cu^{2+} in the pH range preferred by UA-producing lichens (pH 3.5–6;

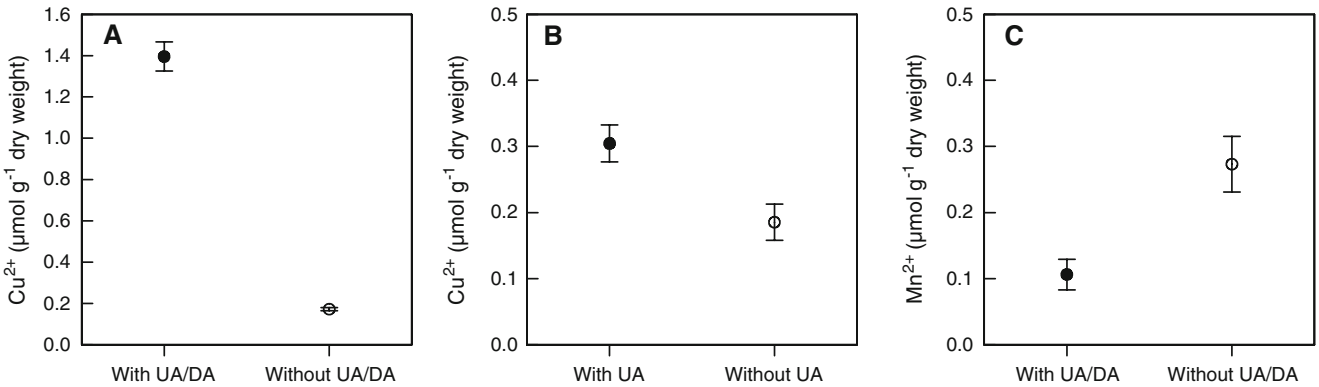

Fig. 1 Intracellular concentrations of Cu^{2+} in **a** *E. mesomorpha* and **b** *R. menziesii* and **c** Mn^{2+} in *E. mesomorpha* with and without their natural content of lichen substances after exposure to $CuCl_2$ or $MnCl_2$ solution for 30 min ($N=5$). *Error bars* indicate standard error

Takani et al. 2002; Hauck and Jürgens 2008; Hauck et al. 2009) suggests that these complexes are involved in the observed promotion of Cu^{2+} uptake. Whether such lipophilic complexes directly cross the phospholipid membrane (as proven for uncomplexed UA; Abo-Khatwa et al. 1996; Hauck and Jürgens 2008) or Cu^{2+} ions are transferred to transporters is unclear. Given the multitude of compounds and their widespread ability to interact with metal ions (Hauck and Huneck 2007), it is likely that promotion of nutrient uptake will be seen in other lichen symbioses. Recent UV-spectroscopic studies in yellow and orange lichen substances, including UA, suggested that promotion of metal uptake by lichen substances might be widespread (Hauck et al. 2009). Lichen compounds that occur in lichens of nutrient-poor sites form complexes with metal ions precisely at the pH ranges preferred by the respective lichens. Lichens growing under pH conditions, at which lichen substances do not bind to metals, are limited to substrata rich in mineral nutrients (Hauck et al. 2009). Research is necessary to elucidate the extent to which lichen substances are involved in the metal homeostasis of lichens.

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Assessment of the Allelopathic Potential of *Juniperus ashei* on Germination and Growth of *Bouteloua curtipendula*

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Abstract Potential allelopathic compounds of *Juniperus ashei* Buchh. (Ashe juniper) and their effect on a native grass were determined in laboratory and field studies. Solid-phase microextraction and gas chromatography/mass spectrometry were used to determine if monoterpenes found in the essential oils of *J. ashei* are released in leaf and litter leachate, as well as volatilized from leaf tissue. Camphor, bornyl acetate, and limonene were found in leaf and fresh litter leachates; however, degraded litter did not contain any of these compounds. Camphor was the most common potentially allelopathic compound found in *J. ashei* leaf and litter leachate and in volatiles from leaf tissue. The effects of leaf and litter tissue on the germination of *Bouteloua curtipendula* (Michx.) Torr. (side-oats grama) was tested by using the "sandwich agar method". The highest germination of *B. curtipendula* (29.6%) occurred in the control, which was significantly higher than fresh litter (13.2%) and degraded litter (16.2%). The lowest germination (6.2%) occurred with *J. ashei* leaves. In the field experiment, aboveground dry mass of *B. curtipendula* was evaluated in relation to position within the canopy and intercanopy of *J. ashei* adult trees when light and water were held constant across locations. Aboveground dry mass of *B. curtipendula* was significantly greater in the intercanopies of *J. ashei* (163.7 g m^2) compared to the dry mass in the understory (44.8 g m^2) and dripline (44.5 g m^2), suggesting some negative influence by *J. ashei*. Chemical analyses indicate that monoterpenes are released through leaching and volatilization from *J. ashei*, and germination and field studies suggest that these compounds inhibit *B. curtipendula*.

Keywords Agar sandwich method · Allelopathy · Ashe juniper · Side-oats grama · Solid-phase microextraction

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Introduction

Juniperus ashei Buchh. (Ashe juniper) is native to the limestone slopes of Central Texas, USA (Bryant and Shafer 1977; Adams 2004). Co-occurring over much of *J. ashei*'s range is the native grass *Bouteloua curtipendula* (Van Auken 2008; Van Auken and McKinley 2008). Grass and forb production and species diversity are greatly reduced under the canopy of *J. ashei* (Schott and Pieper 1985; Wayne and Van Auken 2008). This could be due to competition for water, nutrients, and sunlight. The possibility of an allelopathic effect between *J. ashei* and neighboring plants has been implied (Jameson 1966, 1967, 1970), but not thoroughly investigated.

Allelopathic substances may be released by a variety of mechanisms from various plant structures (see Chou and Patrick 1976; Barney et al. 2005; Nichida et al. 2005; Bonanomi et al. 2006; Gawronska and Golisz 2006 for a review). From the emanation of allelochemicals, plants can regulate the soil microbial community in their immediate vicinity, affect herbivory, encourage beneficial symbiosis, change the chemical and physical properties of the surrounding environment, and directly inhibit the growth of competing plant species (Pedrol et al. 2006). Allelopathic compounds play important roles in the determination of plant diversity, dominance, succession, and climax of natural vegetation and in the plant productivity of agroecosystems (Chou 1999). Allelopathy also may be one of

several attributes which enable a plant to establish in a new ecosystem (Bais et al. 2003; Callaway and Aschehoug 2000; Kilronomos 2002; Callaway and Ridenour 2004) although this has been debated (Blair et al. 2006). Additionally, allelopathy is important in establishing and maintaining community boundaries (Richardson and Williamson 1988; Weidenhamer and Romeo 1989; Williamson et al. 1991).

The purpose of this research was to: (1) determine if the most abundant monoterpenes (camphor, bornyl acetate, and limonene) found in the essential oils of *J. ashei* leach from leaf tissue and from fresh and degraded litter, (2) determine if these monoterpenes are volatilized from leaves of *J. ashei* and if temperature has an effect on the relative abundance of the monoterpenes, (3) determine the effect of *J. ashei* leaves and litter on the germination of *B. curtipendula* (side-oats grama), and (4) evaluate the growth of *B. curtipendula*, in relation to the proximity of mature *J. ashei* trees, when water and light were held constant.

Materials and Methods

Solid-Phase Microextraction and Chromatography New techniques such as solid-phase microextraction (SPME) have proven promising in tracking compounds within plants (Loi et al. 2008). Chromatograms, in combination with the mass spectra, were used to establish presence or absence and relative abundance of limonene, camphor, and bornyl acetate in representative leachate and headspace samples.

Capturing natural rainfall and throughfall was the method of choice. However, the lack of precipitation events during the study period led to creation of an alternative method of obtaining leachate. Litter leachate was created from fresh recently fallen litter, or from aged degraded litter. Samples containing 100 g of each litter type were collected from underneath mature *J. ashei* trees located in a natural area on the University of Texas at San Antonio campus. Geologically, the campus lies on the Balcones Escarpment, an area that defines the southern boundary of the Edwards Plateau of Central Texas. The litter samples were placed in separate beakers that contained 1 l of deionized water. Each beaker was covered with Parafilm™, placed on a stir plate, and stirred for 12 h. A 12-h time frame was chosen to approximate a precipitation event during which litter accumulates in flat areas underneath the canopy and water pools. A fresh branch of *J. ashei* was collected from a mature tree located in the same natural area on the campus of the University of Texas at San Antonio, and approximately 100 g were placed in a beaker that contained 1 l of deionized water. Parafilm™ was placed over the mouth of the beaker, and it was left undisturbed at room temperature (23°C) for 12 h. Litter and leaf material was not ground or fragmented before water extraction. Resulting leachates were filtered to 25 μm by using a sieve, poured into amber bottles, and placed in the refrigerator at 6°C.

Taking into consideration the size, volatility, and polarity of camphor, bornyl acetate, and limonene, Supelco SPME portable field samplers that contained 75-μm-thick fiber coated with Carboxen™/polydimethylsiloxane as the stationary phase (no. 504831, Supelco, Sigma-Aldrich Co., Bellefonte, PA, USA) were chosen. The SPME fibers were conditioned initially for 75 min at 300°C, following manufacturer's directions. Standards and leachate samples were prepared and analyzed with gas chromatography coupled with a mass spectrometer (GC/MS).

For aqueous extraction, 25 ml of the appropriate aqueous sample (standard or leachate) were placed into a 40-ml vial with septum cap and brought to room temperature (23°C). The septum was pierced with the SPME needle, and the fiber was injected into the sample. The fiber was exposed and submerged in the aqueous sample for 10 min while the sample was stirred continually to enhance extraction and reduce extraction time. For headspace extraction, 25 ml of the appropriate aqueous sample were placed into a 40-ml vial with septum cap. Deionized water was used as a control. The vials were heated to 40°C in a water bath. The fiber was exposed in the headspace above the aqueous sample and remained in the headspace of the vial for 15 min, while the sample was continually stirred to enhance extraction and reduce extraction time. The fiber was then retracted into the needle sleeve, withdrawn from the sample vial, and introduced into the GC/MS. The fiber was desorbed and reconditioned by leaving it in the injection port at 300°C for 15 min after each injection.

The compounds volatilized from fresh *J. ashei* leaves at 6°C (refrigerated), 23°C (room temperature), and 60°C (oven) were extracted by placing a branch of fresh leaves in a tightly sealed Reynolds Oven Roasting Bag® (Dalusky, personal communication) that was then held at the appropriate temperature for 4 h. An empty bag was sampled as a control. The first two temperatures tested were chosen because they are the average minimum temperatures recorded in the area for the months of February (6°C) and August (23°C; NOAA 2004). The soil surface temperatures in August consistently reached a temperature of 60°C (data were collected by using Spectrum Watchdogs®, but are not presented); therefore, volatilization at this elevated temperature was considered. The bag was pierced with the SPME needle, and the fiber was exposed for 15 min. The fiber was then retracted into the needle sleeve, withdrawn from the sample, and introduced into the GC/MS. It was desorbed and reconditioned by leaving it in the injection port at 300°C for 15 min after each injection.

Compound desorption and analyses were carried out on a Thermo Finnigan Trace GC gas chromatograph with a

Restek RTX-5MS 30-m×0.5-mm×0.25-μm capillary column coupled with a Finnigan Polaris Q ion trap spectrometer. Splitless injection was employed with an injection temperature of 300°C and ion source temperature of 280°C. The column temperature was programmed to rise from an initial temperature of 60°C to 200°C at a rate of 3°C min^{-1}. Helium was used as carrier gas with a flow rate of 1 ml min^{-1}. A full-scan mass spectrum was obtained and compared to that of commercially purchased and prepared standards of camphor, bornyl acetate, and limonene.

Laboratory Germination Procedures *J. ashei* leaves, freshly fallen litter (fresh litter), and degraded litter were collected from randomly selected trees on the University of Texas at San Antonio campus. Leaves or litter were placed between two layers of agar using the "sandwich method" (Fujii et al. 2004). Purified commercially available agar was prepared at 0.5% *w/v* and autoclaved at 115°C for 15 min. Fresh, clipped *J. ashei* leaves (2.5 g), fresh litter (2.0 g), or degraded litter (2.0 g) was spread in a thin layer in the bottom of 10 (100×15 mm) Petri plates. Fresh litter was distinguished from degraded litter by its recent deposition, surface location, and golden color. Ten Petri plates were prepared without leaves or litter and designated as a control. Twenty milliliters of autoclaved agar were added to each plate. After gelatinizing the agar at room temperature (25°C), another 20 ml of autoclaved agar were dispensed on top of the previous agar layer in each plate creating an "agar sandwich". Seeds of *B. curtipendula* were purchased from Native American Seed Company, Junction, TX, USA (http://www.seedsource.com/). Seeds were collected from the company farms located on the South Llano River just outside the town of Junction, TX, USA. Fifty seeds of *B. curtipendula* were placed onto each of ten leaf, fresh litter, degraded litter, and control agar sandwiches resulting in ten replicates for each agar type. All plates were placed in ambient light and temperature (~23°C) in the laboratory. Germination (rupture of seed coat and emergence of radicle) was determined daily for 14 days.

Percent germination of *B. curtipendula* was analyzed statistically by using analyses of variance with treatment (leaf, fresh litter, degraded litter, and control) as the main effect. Shapiro–Wilk *W* test was used to evaluate normality and the Bartlett's test for homogeneity of variance (Sall et al. 2005). Data were not normal; therefore, percent germination data were arc-sine transformed. Transformed data were normal and variances equal. Tukey–Kramer honestly significant difference (HSD) was used to compare means (Sall et al. 2005).

Field Assessment Methods A field site was established at Government Canyon State Natural Area. Government Canyon State Natural Area is an approximately 3,489 ha site in northwestern Bexar County Texas, just outside of San Antonio and within the Edwards Plateau Region of Central Texas. Aboveground dry mass of *B. curtipendula*, in relation to proximity to *J. ashei*, was evaluated in the field by establishing *B. curtipendula* at three locations associated with mature *J. ashei* trees—at the drip line (edge of the tree), understory (halfway between the trunk and the drip line), and in an intercanopy area (2–3 m away from the drip line). Trees were selected based on size (>50 cm trunk diameter), canopy diameter (minimum of 5 m), and adequate intercanopy space. All planting locations were located on the NW to SW side of the trees. *B. curtipendula* was planted in 20×50-cm (0.1 m^2) plots in nine understory locations, nine drip-line locations, and nine intercanopy locations resulting in a total of 27 plots. Debris, existing vegetation, and existing litter were removed from each plot. Soil was loosened to a depth of 2 cm, smoothed, and leveled. Fifteen grams of *B. curtipendula* seeds were scattered evenly across each plot and pressed lightly into the soil. To reduce the possibility of seed predation or herbivory, wire exclosures were constructed around each plot, using 1×1-cm wire mesh. To minimize variance in ambient temperatures and light between plot locations, the intercanopy plots were draped with shade cloth. To compare light levels at the various locations and to permit the use of light level as a covariate in the analysis of variance if necessary, Spectrum Watch Dogs® were set to record photosynthetically active photon flux density (PFD) per plot location on a clear sunny day from sunrise to sunset. No significant differences in light levels were found among the locations.

The study site preparation began on April 8, 2006, and planting occurred on April 12, 2006. All plots were watered a minimum of once daily to keep the soil moist and promote germination. Prior to establishment, watering was increased sometimes to twice daily when the temperature was high and humidity was low. After establishment and when natural precipitation did not occur at an amount and frequency adequate for viability, the plots were watered manually. Water was sprayed with a hose-end sprayer into the overlying tree canopy allowing the drip to fall onto the plots. The planting plots continued to be observed daily and watered as necessary through October 14, 2006 at which time the aboveground (flower + stem + leaf) biomass was harvested. Aboveground total dry mass was determined by drying at 100°C to constant mass and weighing.

Early in the study, it was observed that germination of one of the intercanopy plots was almost nonexistent, lagging far behind the other plots. Rabbit droppings were routinely found on top of the exclosure. A decision was made to remove this plot from the statistical analysis. Analysis of variance of aboveground total dry mass was performed with location (understory, dripline, intercanopy)

as the main effect in the model. In addition, the mean daily PFDs for each position were entered into the model as a covariate. Photosynthetically active photon flux density was not a significant factor (i.e., light levels were not significantly different among the locations) and was removed as a covariate from the model. The Shapiro–Wilk *W* test was used to check for data normality, and the Bartlett's test was used to test for homogeneity of variance. Due to unequal variances, aboveground dry mass was log transformed. A Tukey–Kramer HSD comparison for all pairs was performed.

Results

Solid-Phase Microextraction and Chromatography The approximate retention time of camphor was 14.72 min, of bornyl acetate was 20.75 min, and of limonene was 9.66 min. Table 1 indicates the occurrence of the analytes in the representative samples. The results of the GC/MS analysis of the aqueous leachate confirm the presence of camphor, bornyl acetate, and limonene in leachate derived from fresh leaves and fresh litter but not in leachate derived from degraded litter (Table 1). When analyzing leaf- and fresh litter leachate for the three compounds, camphor was the most abundant, bornyl acetate was intermediate, and limonene was the least abundant.

The results of the GC/MS analysis of the headspace over aqueous leachate samples derived from fresh *J. ashei* leaves and fresh *J. ashei* litter confirm the presence of all three compounds in the headspace, but not in the headspace of samples of leachate derived from degraded litter (Table 1). With leaf tissue, camphor was the most abundant, bornyl acetate was intermediate, and limonene was the least abundant; however, with fresh litter, camphor was the most abundant, followed by limonene, and only a trace of bornyl acetate.

Table 1 Occurrence of camphor, bornyl acetate, and limonene in leachate and volatilized headspace samples analyzed using solid phase microextraction coupled with GC/MS

Sample type	Camphor	Bornyl acetate	Limonene
Aqueous submerged			
Juniperus ashei leaves	1	2	3
Juniperus ashei fresh litter	1	2	3
Juniperus ashei degraded litter	0	0	0
Aqueous headspace			
Juniperus ashei leaves	1	2	3
Juniperus ashei fresh litter	1	TR	2
Juniperus ashei degraded litter	0	0	0
Volatilized (headspace)			
6°C	2	1	3
23°C	1	2	3
60°C	1	3	2

Abundance ranking are 0—absent, 1—highest, 2—second highest, 3—least abundant
TR trace (present but in amounts <1%)

The results of the GC/MS analysis of the compounds volatilized from fresh *J. ashei* leaves at 6°C (refrigerated), 23°C (room temperature), and 60°C (oven) confirm the presence of camphor, limonene, and bornyl acetate in all samples (Table 1; Fig. 1). The relative abundance of bornyl acetate was the highest at 6°C and decreased as the temperature rose. Bornyl acetate became the least abundant relative to camphor and limonene when the temperature reached 60°C. The relative abundance of camphor increased with increasing temperature. Amounts of limonene ranked relatively low in all samples but surpassed that of bornyl acetate when the temperature reached 60°C (Table 1; Fig. 1).

Laboratory Germination Experiment Analysis of variance indicated a significant difference between mean germination (arcsine transformation) of *B. curtipendula* ($P<0.001$). The highest germination of *B. curtipendula* (29.6%) occurred in the control, which was significantly greater than the fresh litter treatment (13.2%) and the degraded litter treatment (16.2%; Fig. 2). The lowest germination (6.2%) occurred in the leaf treatment, which was significantly lower than all other treatments (Fig. 2).

Field Experiment Analysis of variance of *B. curtipendula* aboveground dry mass indicated a significant difference between locations ($P=0.002$). The highest mean aboveground dry mass occurred in the intercanopy locations (163.7 g m^{-2}) which was significantly greater than the mean of both the understory locations (44.8 g m^{-2}) and the dripline locations (44.5 g m^{-2}; Fig. 3). The mean aboveground dry mass of the understory and dripline plots were not significantly different from each other.

Discussion

Studies have identified camphor at 68.5%, bornyl acetate at 12.2%, and limonene at 5.8% in the essential oils of *J. ashei* acquired through steam distillation (Adams 2000). However, to our knowledge, it has not been shown that these compounds can be leached from leaf tissue or litter, and/or volatilized from the leaves of *J. ashei*, or other species of *Juniperus*. Studies have noted suppression of understory vegetation beneath *Juniperus* species (Lavin et al. 1968; Fuhlendorf et al. 1996; Wayne and Van Auken 2008), and while the role of allelopathy has been investigated for some species of *Juniperus* (Jameson 1966, 1970; Lavin et al. 1968; Horman and Anderson 2003), no studies have shown allelopathy by *J. ashei*.

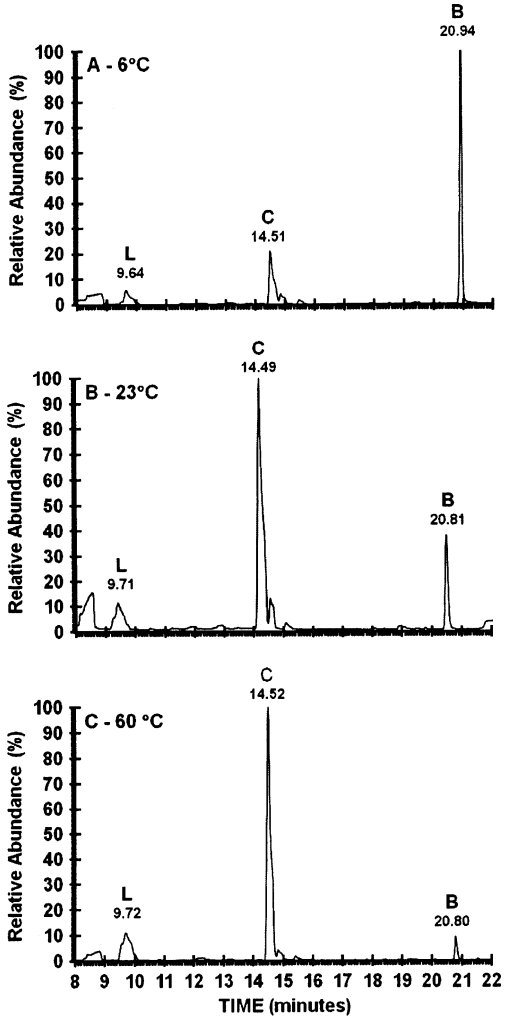

Fig. 1 Gas chromatogram of the headspace within a sealed Reynolds Oven Roasting Bag® containing a fresh branch of *J. ashei* held for 4 h at **a** 6°C, **b** 23°C, or **c** 60°C. *L* limonene, *C* camphor, *B* bornyl acetate. The *number* indicates time

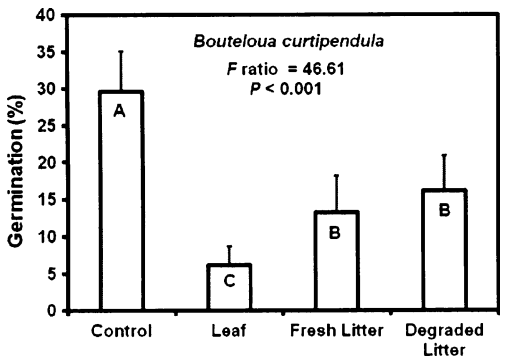

Fig. 2 Percent germination of *B. curtipendula* on agar (control), agar with leaf tissue, fresh litter, and degraded litter. *Error bars* represent one standard deviation. Means with the *same letter* are not significantly different ($P>0.05$; Tukey–Kramer HSD)

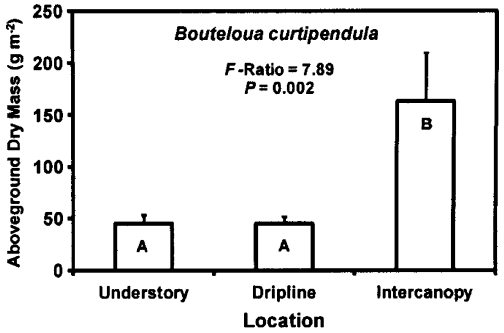

Fig. 3 Aboveground dry mass of *B. curtipendula* per m^{-2} for each location (understory, dripline, and intercanopy). *Error bars* represent one standard error of the mean. Means with the *same letter* are not significantly different ($P>0.05$, Tukey–Kramer HSD)

Essential oils and individual monoterpenes, such as camphor, bornyl acetate, and limonene, strongly inhibit seed germination and plant growth (Duke and Olivia 2002; Barney et al. 2005). Research has shown that even dilute unsaturated solutions of monoterpenes may act as potent biological inhibitors (Weidenhamer et al. 1993). Additionally, the activity of monoterpenoids has been shown to be species selective (Williamson et al. 1992; Abrahim et al. 2000; Vokou et al. 2003; Loi et al. 2008)

There is evidence that monoterpenes inhibit mitosis, inhibit cell nucleus and organelle DNA synthesis, and cause deterioration of membrane integrity (Dayan et al. 2000; Romagni et al. 2000; Duke and Olivia 2002). Lipophilic monoterpenes cause dewaxing and induce stomatal opening, which causes increased transpiration and death of *Arabidopsis thaliana* (Schulz et al. 2007). The molecular mode of action of most monoterpenes is not well investigated, and we did not look at mode of action in this study.

Characteristics of monoterpenes that affect their volatilization, such as vapor pressure, have been evaluated at various temperatures (Li and Perdue 1998). Similar to this study where bornyl acetate seemed to be the compound most affected by changes in temperature, *Psuedotsuga menziesii* leaves from plants grown at different temperatures showed reductions in the concentration of bornyl acetate but no changes in camphene or limonene at elevated temperatures (Snow et al. 2003). Apparent higher total terpene emission from *Rosmarinus officinalis* plants when ambient air temperatures were the highest were not significant in a previous study (Peñuelas and Llusià 1997). It is unclear whether the variations in relative abundance in this study are due to chemical properties of the compounds, metabolic properties, or something else.

While no camphor, bornyl acetate, or limonene was detected in the aqueous sample or headspace of degraded litter, germination of *B. curtipendula* on the degraded "sandwich" agar treatment was not significantly different from fresh litter samples. A fungal growth developed on

seeds in the degraded litter samples during the germination portion of the study. This fungus was not present in any of the other treatments. Decreased germination in the degraded litter sample could be due to fungal effects on seeds. Fungi can have saprophytic or pathogenic effects on seeds (Christensen 1989), and fungal seed pathogens may inhibit or stimulate seed germination (Harman 1983).

The overall effect of one plant on another is the result of multiple interacting mechanisms, and resource competition should always be considered. Therefore, our experimental design adjusted for anticipated light and water differences. The results indicated that the difference in aboveground dry mass was not due to light or water. Other factors that may have contributed to the differences in aboveground dry mass, however, include temperature, pH, nutrient availability, soil microfauna, microbiological differences, and the presence of allelopathic compounds introduced by *J. ashei* (Callaway 1995; Gawronska and Golisz 2006).

In early successional xeric, semiarid, and arid savannas, soil C and N are the highest underneath the canopy of shrubs (Bush 2008). In mesic *J. virginiana* and associated grassland communities, ecosystem C and N stocks revealed significant C and N accrual in both plant biomass and soils in newly established *J. virginiana* forests (McKinley and Blair 2008). Based on these studies, we hypothesize that nutrient levels would be higher underneath the canopy, resulting in greater growth of *B. curtipendula* beneath the canopy and at the dripline. This is contrary to our findings, thus suggesting that the significant difference in aboveground dry mass observed among locations is probably due to a factor other than C and N availability.

Leachates in this study were generated artificially due to lack of precipitation events, and we did not quantify the concentrations of camphor, bornyl acetate, and limonene released through leaching or volatilization; however, our results show that all three chemicals are released into the environment from *J. ashei*. Combination of the chemical analyses with the germination and field studies strengthens the hypothesis that allelopathic compounds may be influencing germination and growth of *B. curtipendula*.

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Identification of Sex Pheromone Components of the Hessian Fly, *Mayetiola destructor*

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Abstract Coupled gas chromatographic (GC)–electroantennographic detection (EAD) analyses of ovipositor extract of calling Hessian fly, *Mayetiola destructor*, females revealed that seven compounds elicited responses from male antennae. Four of the compounds—(2*S*)-tridec-2-yl acetate, (2*S*,10*Z*)-10-tridecen-2-yl acetate, (2*S*,10*E*)-10-tridecen-2-yl acetate, and (2*S*,10*E*)-10-tridecen-2-ol—were identified previously in female extracts. Two new EAD-active compounds, (2*S*,8*Z*,10*E*)-8,10-tridecadien-2-yl acetate and (2*S*,8*E*,10*E*)-8,10-tridecadien-2-yl acetate, were identified by GC–mass spectroscopy (MS) and the use of synthetic reference samples. In a Y-tube bioassay, a five-component blend (1 ng (2*S*)-tridec-2-yl acetate, 10 ng (2*S*,10*E*)-10-tridecen-2-yl acetate, 1 ng (2*S*,10*E*)-10-tridecen-2-ol, 1 ng (2*S*,8*Z*,10*E*)-8,10-tridecadien-2-yl acetate, and 1 ng (2*S*,8*E*,10*E*)-8,10-tridecadien-2-yl acetate) was as attractive to male Hessian flies as a similar amount of female extract (with respect to the main compound, (2*S*,10*E*)-10-tridecen-2-yl acetate). The five-component blend was more attractive to male flies than a three-component blend lacking the two dienes. Furthermore, the five-component blend was more attractive than a blend with the same compounds but that contained one tenth the concentration of (2*S*,8*E*,10*E*)-8,10-tridecadien-2-yl acetate (more accurately mimicking the ratios found in female extract). This suggests that the ratios emitted by females might deviate from those in gland extracts. In a field-trapping experiment, the five-component blend applied to polyethylene cap dispensers in a 100:10 μg ratio between the main component and each of the other blend components attracted a significant number of male Hessian flies. Also, a small-plot field test demonstrated the attractiveness of the five-component blend to male Hessian flies and suggests that this pheromone blend may be useful for monitoring and predicting Hessian fly outbreaks in agricultural systems.

Keywords *Mayetiola destructor* · Hessian fly · Cecidomyiidae · Diptera · Sex pheromone · (2*S*)-Tridec-2-yl acetate · (2*S*,10*E*)-10-tridecen-2-yl acetate · (2*S*,10*E*)-10-Tridecen-2-ol · (2*S*,8*Z*,10*E*)-8 · 10-Tridecadien-2-yl acetate · (2*S*,8*E*,10*E*)-8 · 10-Tridecadien-2-yl acetate · GC–EAD · Y-tube bioassay · Field trapping

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Introduction

The Hessian fly, *Mayetiola destructor* (Say) (Diptera: Cecidomyiidae), is one of the most destructive pests of wheat (*Triticum* spp) in the US and North Africa (Gagné 1989; El Bouhssini et al. 1999; Berzonsky et al. 2003; Harris et al. 2003). In addition, it is considered a pest in

many European countries (Barnes 1956). In common with other species in the family Cecidomyiidae (gall midges), Hessian fly adults are short-lived, have a highly synchronized period of flight activity, and may be present in crops for brief periods of time only (Harris and Foster 1999). Typically, outbreaks are sporadic, local, and difficult to predict (*ibid*.). As a consequence, Hessian flies are normally discovered only after they have become a serious problem.

Various methods are used to control Hessian flies including delayed planting of winter wheat, use of resistant wheat varieties, and application of broad-spectrum insecticides (Buntin et al. 1992; El Bouhssini et al. 1999; Rausher 2001; Berzonsky et al. 2003). All of these methods might be improved by a sensitive method for the detection of early infestations (Buntin et al. 1990, 1992; Cox and Hatchett 1986; Harris et al. 2003). One potential method for field monitoring might employ the use of sex pheromone-baited traps (Harris and Foster 1999). Earlier work demonstrated the presence of a sex pheromone in the Hessian fly (McKay and Hatchett 1984). One component of the female-produced sex pheromone, (2*S*,10*E*)-10-tridecen-2-yl acetate [2*S*-10*E*-13:OAc], was identified (Foster et al. 1991b). However, when tested alone in a wind tunnel, this compound attracted significantly fewer Hessian fly males than did female extract (Harris and Foster, 1991). In a field study, traps baited with the compound also failed to attract male Hessian flies (Harris and Foster 1999). In the wind tunnel, male responses were tested to binary blends of 2*S*-10*E*-13:OAc and racemic mixtures of three other chemicals—(10*Z*)-10-tridecen-2-yl acetate, (10*E*)-10-tridecen-2-ol, and tridec-2-yl acetate (Foster et al. 1991b; Harris and Foster 1991)—found in female extract (Millar et al. 1991). However, none of these blends attracted more males than 2*S*-10*E*-13:OAc alone. In this study, we identified additional compounds produced by female Hessian fly and formulated a blend that is attractive to male flies in the field.

Methods and Materials

Insect Rearing Wheat plants (*Triticum aestivum* L., genotype "Blueboy") that contained Hessian fly puparia of the "Great Plains" biotype (provided by the US Department of Agriculture-Agricultural Research Service (USDA-ARS) and the Department of Entomology, Purdue University, West Lafayette, IN, USA) were put into Plexiglas cages (29×34×29 cm) held in an environmental chamber (25°C, 70% relative humidity (RH) and 12:12 L:D photoperiod; lights on 09:00) at Alnarp, Sweden. Infested plants were watered daily to avoid desiccation of developing flies. Typically, adults emerged 7–14 days later, and the sexes were separated and used for pheromone extraction, coupled gas chromatographic–electroantennographic detection (GC–EAD) analyses, and Y-tube bioassays. Hessian flies used in the small-plot test were reared on hard red spring wheat, *T. aestivum* L. (genotype "Reeder"), in a greenhouse at North Dakota State University. These flies were also of the "Great Plains" biotype and originated from approximately 5,000 puparia, obtained in 2000, from the USDA-ARS laboratory at Purdue University.

Pheromone Extraction The pheromone gland in Hessian fly females is associated with the eighth and ninth abdominal intersegmental membrane epidermis (Solinas and Isidoro 1996). Gland extracts were prepared by excising the terminal abdominal segments of virgin calling females (Bergh et al. 1990; Foster et al. 1991a). Ovipositors were placed into a vial partially immersed in liquid nitrogen. When sufficient ovipositors (approximately 30) were collected, the vial was allowed to warm to ambient temperature, and the tissue was extracted for 1–1.5 min in distilled hexane (LabScan). Following extraction, the solvent was decanted into glass vials (2 ml) and stored at −18°C until use. All dissections were made between 09:00 and 11:30.

GC–EAD Recordings GC–EAD (Arn et al. 1975) was used to analyze female gland extracts and synthetic compounds. A Hewlett-Packard 6890 GC (Palo Alto, CA, USA) with flame ionization detection and an Innowax column (30 m× 0.25 mm i.d., H-P), programmed from 80°C (held for 2 min) to 220°C at 10°C/min, was used. Whole male bodies were mounted in an antennal holder (JoAC, Lund, Sweden), as described by Hillbur et al. (2001). Both antennae were exposed simultaneously to a charcoal-filtered and humidified air stream at a rate of approximately 0.3 m/s through a glass tube (8-mm diameter). The signals from the antennae were amplified (JoAC) before they were recorded and were analyzed with ElectroAntennoGraphy software (Syntech, Hilversum, The Netherlands).

Structure Elucidation Coupled GC–MS analyses of extracts of female Hessian fly were generally carried out as described earlier (Hillbur et al. 2005). Separations were achieved with a 60 m, 0.25-mm i.d. fused silica capillary, DB-5/MS (J&W Scientific, Folsom, CA, USA) under temperature program: 3 min at 60°C, then programmed to 280°C at a rate of 5°C/min. An additional fused silica column, 50 m, 0.25 mm i.d., Permabond FFAP (Macherey & Nagel, Düren, Germany), was also used.

Enantioselective GC was carried out with a 25 m, 0.25-mm i.d. fused silica capillary coated with a 1:1 mixture of heptakis 6-*O*-*tert*.butyldimethylsilyl-2,3-di-*O*-methyl β-cyclodextrin and OV 17 at 100°C isothermal and using hydrogen as the carrier gas.

^{1}H nuclear magnetic resonance (NMR) spectra of synthetic compounds were recorded on a Bruker AMX-400 instrument (Karlsruhe, Germany) using tetramethylsilan as the internal standard.

Syntheses If not otherwise stated, starting material, reagents, and solvents were purchased from Aldrich and were of highest available grade. Syntheses were carried out under dry argon and synthetic compounds were purified by column chromatography using silica 60 Å, 32–63 mesh (MP Eco Chrom) under 0.5-bar pressure.

The stereogenic center in each of the six target compounds was introduced via a Grignard-type reaction of a suitable precursor with commercially available (2*S*)-2-methyloxiran of 98% optical purity similar to the approach of Millar et al. (1991). The enantiomeric purity of the products corresponded with commercial (2*S*)-2-methyloxiran (checked by enantioselective GC). The Grignard reagent was produced from commercially available 1-bromodecane and used to produce (2*S*)-tridec-2-yl acetate [2*S*-13:OAc]. Synthesis of the mono-unsaturated compounds (i.e., 2*S*-10*E*-13:OAc, (2*S*,10*E*)-10-tridecen-2-ol [2*S*-10*E*-13:OH], and (2*S*,10*Z*)-10-tridecen-2-yl acetate [2*S*-10*Z*-13:OAc]) involved 7-decyn-1-ol as the educt (Yadav et al. 1995). Reduction of the ynol to (7*E*)-7-decen-1-ol (Rossi and Carpita 1977) or to (7*Z*)-7-decen-1-ol (hydrogenation over Lindlar catalyst), transformation to the corresponding alkenyl bromides (Wiley et al. 1964), and formation of the Grignard products followed by chain elongation with (2*S*)-2-methyloxiran furnished 2*S*-10*E*-13:OH and its (*Z*)-isomer in good yields and purities of approximately 96–98%. Standard laboratory acetylation produced the corresponding acetates. Analytical data of all these known compounds were in good accord with expected values and those reported in the literature (Millar et al. 1991; Yadav et al. 1995).

The scope of the syntheses of (2*S*,8*Z*,10*E*)-8,10-tridecadien-2-yl acetate [2*S*-8*Z*-10*E*-13:OAc] and (2*S*,8*E*,10*E*)-8,10-tridecadien-2-yl acetate [2*S*-8*E*-10*E*-13:OAc] is shown in Figs. 3 and 4, respectively.

The synthesis of 2*S*-8*Z*-10*E*-13:OAc (Fig. 3) started from commercially available 5-bromopentan-1-ol (**I**), which was converted to the silyl ether **II** and subsequently transformed to the Wittig reagent using triphenyl phosphane. Wittig reaction with (2*E*)-2-butenal yielded **III**. Deprotection and transformation of the obtained alcohol to the corresponding bromide (Horner et al. 1959) produced (5*Z*,7*E*)-5,7-decadien-1-yl bromide (**IV**). Chain elongation with (2*S*)-2-methyloxiran followed by acetylation afforded the target compound 2*S*-8*Z*-10*E*-13:OAc.

The synthesis of 2*S*-8*E*-10*E*-13:OAc (Fig. 4) started from commercially available 4-bromo-1-butene (**V**). Chain elongation with (2*S*)-2-methyloxiran furnished (2*S*)-7-hepten-2-ol. Reaction with 3,4-dihydro-2*H*-pyran and ozonization followed by reductive workup produced (5*S*)-5-(2-tetrahydropyranyloxy)hexan-1-ol (**VII**) via the protected alkenol **VI**. Transformation of the primary hydroxyl group to the bromide **VIII** and chain elongation of its Grignard product with (2*E*,4*E*)-2,4-heptadien-1-yl acetate (Samain et al. 1978) employing cuprate coupling (Fouquet and Schlosser 1974) yielded (2*S*,8*E*,10*E*)-2-(2-tetrahydropyranyloxy)trideca-8,10-diene (**IX**). Acid-catalyzed deprotection and acetylation afforded the target compound 2*S*-8*E*-10*E*-13:OAc.

*5-Bromo-1-tert.butyldimethylsilyloxypentane (**II**)* A solution of 5.70 g (34.1 mmol) 5-bromopentane-1-ol (**I**; purchased from TCI Europe, Zwijendrecht, Belgium) and 6.18 g (90.9 mmol) imidazole in 5 ml of abs. dimethylformamide was cooled to 0°C. After addition of 6.84 g (45.5 mmol) *tert*.butyldimethylsilylchloride, the mixture was stirred for 3 h, warmed to 10°C, and stirred for an additional 2 h. Workup started with the addition of 150 ml diethyl ether and 200 ml of saturated aqueous sodium bicarbonate, separation of the organic layer, and extraction of the aqueous layer (3×150 ml diethyl ether). The organic solutions were combined, washed with brine, and dried over magnesium sulfate. After removal of the solvent, the residue was chromatographed on silica using hexane–diethyl acetate 95:5, yielding 7.25 g (25.7 mmol, 75%) of **II** as a colorless liquid.

^{1}H-NMR (400 MHz, $CDCl_3$) δ [ppm] = 0.85 (s, 9H, SiC(CH_3)$_3$); 1.39–1.55 (m, 4H, 2-H, and 3-H); 1.79–1.88 (m, 2H, 4-H); 3.36 (t, 2H, *J*=6.87 Hz, 5-H); 3.57 (t, 2H, *J*=6.11 Hz, 1-H)

^{13}C-NMR (100.6 MHz, $CDCl_3$) δ [ppm] = −5.14 (q, 2C, Si(**C**H$_3$)$_2$); 24.75 (t, 3-C); 26.12 (q, 3C, SiC(*C*H$_3$)$_3$); 32.07 (t, 2-C); 32.78 (t, 4-C); 33.95 (t, 5-C); 63.01 (t, 1-C)

MS (70 eV) m/z [%] = 169 (40); 167 (41); 139 (40); 137 (42); 115 (6); 101 (10); 99 (7); 89 (10); 88 (5); 85 (6); 75 (44); 73 (27); 70 (11); 69 (100); 61 (5); 59 (13); 58 (9); 57 (16); 55 (5); 47 (6); 45 (9); 43 (7); 41 (45); 39 (9)

*(5Z,7E)-5,7-Decadienyloxy-tert.butyldimethylsilane (**III**)* A mixture of 7.18 g (23.6 mmol) of the bromide **II**, 13.4 g (51.1 mmol) triphenyl phosphane, and 813 mg (7.70 mmol) sodium carbonate in 50 ml acetonitrile was refluxed for 24 h. The solvent was removed from the suspension, and the residue was dissolved in 50 ml dichloromethane. Filtration of the oily solution over silica, using dichloromethane as the eluent, provided unreacted starting material, while the Wittig salt (11.0 g, 20.2 mmol, 79%) was isolated using a 1:1 mixture of dichloromethane and methanol as the eluent.

^{1}H-NMR (400 MHz, $CDCl_3$) δ [ppm] = 0.85 (s, 9H, SiC(CH_3)$_3$); 1.50–1.75 (m, 8H, 2-H to 5-H); 3.60 (t, 2H, *J*=6.1 Hz, 1-H); 7.71–7.91 (m, 15H, H_{Ph})

^{13}C-NMR (100.6 MHz, $CDCl_3$) δ [ppm] = −5.54 (q, 2C, Si(CH_3)$_2$); 22.25/27.93/28.09 (t, 3-C to 5-C); 26.10 (q, 3C, SiC(CH_3)$_3$); 32.07 (t, 2-C); 63.43 (t, 1-C); 131.32 (d, 6C, $C_{Ar)}$; 134.57 (d, 9C, C_{Ar}); 136.02 (s, 3C, C_{Ar})

A solution of 10.6 g (20.0 mmol) of the Wittig salt, obtained in the previous step, in 100 ml abs. tetrahydrofuran was cooled to 0°C. Subsequently, 26.2 ml of a solution of potassium hexamethyldisilazane (0.5 mmol in toluene, equal to 3.26 g, 16.3 mmol KHMDS) was added slowly, with stirring continued for 2 h. Subsequently, the mixture was cooled to −78°C and 1.34 g (16.0 mmol); (2*E*)-2-pentenal, dissolved in 20 ml abs. tetrahydrofuran, were added dropwise. After stirring for another half hour, the mixture was warmed to 20°C, and 350 ml of a saturated aqueous solution of ammonium chloride was added. The mixture was extracted five times with 150 ml diethyl ether. The combined organic solutions were washed with brine and dried over magnesium sulfate. Removal of the solvent *in vacuo* and chromatography on silica using hexane–ethyl acetate (97:3) yielded 2.77 g (10.3 mmol, 55%) of the protected dienol **III** as a colorless oil.

^{1}H-NMR (400 MHz, $CDCl_3$) δ [ppm] = 0.94 (t, 3H, *J*= 7.5 Hz, 10-H); 0.99 (s, 9H, SiC(CH_3)$_3$); 1.42–1.57 (m, 4H, 2-H, and 3-H); 1.96–2.05 (m, 2H, 9-H); 2.11–2.25 (m, 2H, 4-H); 3.52 (t, 2H, *J*=6.3 Hz, 1-H); 5.31–5.38 (m, 1H, 5-H); 5.59–5.68 (m, 1H, 8-H); 6.06–6.13 (m, 1H, 6-H); 6.38–6.48 (m, 1H, 7-H)

^{13}C-NMR (100.6 MHz, $CDCl_3$) δ [ppm] = -5.13 (q, 2C, Si(CH_3)$_2$); 13.91 (q, 10-C); 26.20 (q, 3C, SiC(CH_3)$_3$); 26.28 (t, 3-C); 26.44 (t, 9-C); 27.82 (t, 4-C); 32.77 (t, 2-C); 63.13 (t, 1-H); 125.42 (d, 7-C); 129.61 (d, 6-C); 129.92 (d, 5-C); 136.27 (d, 8-C)

MS (70 eV) m/z [%] = 211 (41); 155 (33); 135 (20); 129 (5); 115 (8); 107 (13); 101 (13); 99 (5); 95 (9); 93 (25); 91 (8); 89 (12); 81 (10); 79 (28); 77 (11); 76 (9); 75 (100); 73 (26); 69 (7); 67 (13); 59 (11); 57 (6); 55 (10); 41 (13)

*(5Z,7E)-5,7-Decadienyl bromide (***IV***)* To a solution of 2.77 g (10.3 mmol) **III** in 50 ml tetrahydrofuran was added 14.4 ml (14.4 mmol) of a 1-M solution of tetra-*n*-butylammonium fluoride in tetrahydrofuran, and the mixture was stirred for 6 h at 20°C. Workup started with the addition of 100 ml diethyl ether and 100 ml of a saturated aqueous solution of ammonium chloride, separation of the layers, and extraction of the aqueous layer (6×100 ml diethyl ether). The combined organic layers were washed with brine and dried over magnesium sulfate. Removal of the solvent *in vacuo* and purification by silica chromatography (hexane–ethyl acetate 4:1) gave 1.52 g (9.85 mmol, 96%) (5*Z*,7*E*)-5,7-decadien-1-ol.

^{1}H-NMR (400 MHz, $CDCl_3$) δ [ppm] = 0.68 (br.s, 1H, OH); 0.92 (t, 3H, *J*=7.3 Hz, 10-H); 1.28–1.41 (m, 4H, 2-H, and 3-H); 1.96–2.05 (m, 2H, 9-H); 2.08–2.16 (m, 2H, 4-H); 3.32 (t, 2H, *J*=6.1 Hz, 1-H); 5.27–5.33 (m, 1H, 5-H); 5.59–5.68 (m, 1H, 8-H); 6.05–6.15 (m, 1H, 6-H); 6.37–6.46 (m, 1H, 7-H)

^{13}C-NMR (100.6 MHz, $CDCl_3$) δ [ppm] = 13.91 (q, 10-C); 26.26 (t, 9-C); 26.30 (t, 3-C); 27.81 (t, 4-C); 32.67 (t, 2-C); 62.54 (t, 1-H); 125.38 (d, 7-C); 129.59 (d, 6-C); 129.84 (d, 5-C); 136.32 (d, 8-C)

MS (70 eV) m/z [%] = 154 (5); 136 (14); 121 (7); 111 (6); 108 (8); 107 (14); 98 (9); 97 (9); 95 (28); 94 (10); 93 (33); 91 (20); 84 (7); 83 (14); 82 (15); 81 (30); 79 (100); 78 (9); 77 (27); 72 (6); 71 (7); 70 (16); 69 (13); 68 (24); 67 (81); 66 (9); 65 (12); 57 (8); 56 (5); 55 (33); 54 (8); 53 (14); 51 (5); 43 (6); 41 (24); 39 (14)

A solution of 2.62 g (10.0 mmol) triphenyl phosphane and 680 mg (10.0 mmol) imidazole in a mixture of 50 ml abs. diethyl ether and 2 ml abs. acetonitrile was cooled to 0°C. Subsequently, 1.62 g (10.0 mmol) of bromine was added dropwise, and a yellowish suspension was formed that was stirred for 2 h at 20°C. A solution of 1.52 g (9.85 mmol) of (5*Z*,7*E*)-5,7-decadien-1-ol, obtained in the previous step, in 20 ml abs. diethyl ether was added slowly, and the mixture was stirred overnight. Workup started with the addition of 100 ml diethyl ether and 100 ml of a saturated aqueous solution of ammonium chloride, separation of the layers, and extraction of the aqueous layer (6× 100 ml diethyl ether). The combined organic solutions were washed with brine and dried over magnesium sulfate. After removal of the solvent, the residue was chromatographed on silica (hexane–ethyl acetate 97:3) yielding 1.57 g (7,23 mmol, 73%) of the bromide **IV** as a colorless oil.

^{1}H-NMR (400 MHz, $CDCl_3$) δ [ppm] = 0.92 (t, 3H, *J*= 7.5 Hz, 1-H); 1.17–1.27 (m, 2H, 8-H); 1.43–1.53 (m, 2H, 9-H); 1.92–2.05 (m, 4H, 2-H, and 7-H); 2.91 (t, 3H, *J*= 6.8 Hz, 10-H); 5.13–5.21 (m, 1H, 3-H); 5.58–5.67 (m, 1H, 6-H); 6.01–6.10 (m, 1H, 4-H); 6.29–6.39 (m, 1H, 5-H)

^{13}C-NMR (100.6 MHz, $CDCl_3$) δ [ppm] = 13.88 (q, 1-C); 26.28/27.0 (t, 2-C, and 7-C); 28.37 (t, 8-C); 32.46 (t, 9-C); 33.41 (t, 10-C); 125.15 (d, 5-C); 129.02 (d, 3-C); 129.88 (d, 4-C); 136.64 (d, 6-C)

MS (70 eV) m/z [%] = 218 (11); 216 (11); 109 (9); 107 (7); 96 (7); 95 (100); 93 (13); 91 (10); 82 (17); 81 (48); 80 (7); 79 (26); 77 (15); 69 (7); 68 (23); 67 (66); 65 (7); 55 (18); 53 (9); 41 (23); 39 (13).

2S-8Z-10E-13:OAc To a mixture of 353 mg (14.5 mmol) freshly crushed magnesium turnings and 30 ml abs. tetrahydrofuran kept at 60°C, a solution of 1.57 g of **IV** in 30 ml tetrahydrofuran was added dropwise. The solution of the Grignard product was added dropwise to a suspension of 420 mg (7.23 mmol) of (2*S*)-2-methyloxiran and 140 mg (0.72 mmol) copper-I-iodide in 10 ml abs. tetrahydrofuran that was cooled to −78°C. After warming to room temperature, the mixture was stirred for another hour. Workup started with the addition of 50 ml diethyl ether and 100 ml of a saturated aqueous solution of ammonium chloride, separation of the layers, and extraction of the aqueous layer (4×50 ml hexane–ethyl acetate 1:1). The combined organic solutions were dried over magnesium sulfate and concentrated *in vacuo*. The obtained (2*S*,8*Z*,10*E*)-8,10-tridecadien-2-ol was purified by column chromatography on silica (hexane–ethyl acetate 1:1) yielding 654 mg (3.33 mmol, 46%) of (2*S*,8*Z*,10*E*)-trideca-8,10-dien-1-ol.

^{1}H-NMR (400 MHz, $CDCl_3$) δ [ppm] = 0.93 (t, 3H, *J*= 7.4 Hz, 13-H); 0.99 (d, 3H, *J*=6.1 Hz, 1-H); 1.15–1.39 (m, 9H, 3-H to 6-H and OH); 1.96–2.07 (m, 2H, 12-H); 2.13–2.21 (m, 2H, 7-H); 3.45–3.55 (m, 1H, 2-H); 5.32–5.42 (m, 1H, 11-H); 5.60–5.70 (m, 1H, 8-H); 6.09–6.15 (m, 1H, 10-H); 6.42–6.39 (m, 1H, 9-H)

^{13}C-NMR (100.6 MHz, $CDCl_3$) δ [ppm] = 13.94 (q, 13-C); 23.81 (q, 1-C); 26.03 (t, 12-C); 26.31 (t, 7-C); 28.07/29.61/30.14/39.70 (t, 3-C to 6-C); 67.68 (d, 2-C); 128.22 (d, 9-C); 129.42 (d, 11-C); 130.08 (d, 10-C); 136.26 (d, 8-C)

MS (70 eV) m/z [%] = 149 (7); 135 (7); 125 (13); 122 (5); 121 (13); 114 (8); 111 (8); 110 (16); 109 (13); 108 (14); 107 (25); 100 (9); 97 (14); 96 (52); 95 (59); 94 (18); 93 (83); 91 (20); 83 (12); 82 (52); 81 (63); 80 (26); 79 (91); 78 (11); 77 (23); 71 (14); 69 (11); 68 (17); 67 (100); 66 (11); 65 (10); 57 (6); 55 (31); 54 (9); 53 (13); 45 (48); 43 (19); 41 (33); 39 (14)

A solution of 641 mg (3.26 mol) of the aforementioned tridecadien-2-ol and 10 mg of *N*,*N*-dimethyl-4-aminopyridine in 10 ml abs. pyridine was cooled to 0°C. Subsequently, 916 mg (9.09 mmol) acetic anhydride, dissolved in 5 ml tetrahydrofuran, were added dropwise. After warming to room temperature, the mixture was stirred for 2 h. Workup started with the addition of 50 ml diethyl ether and 100 ml of an ice-cold saturated aqueous solution of sodium bicarbonate, separation of the layers, and extraction of the aqueous layer (five times with 50 ml diethyl ether). The combined organic solutions were washed with 50 ml of an aqueous saturated solution of copper-II-sulfate and 50 ml of brine and dried over magnesium sulfate. After removal of the solvent *in vacuo*, the crude product was chromatographed on silica (hexane–ethyl acetate 95:5), yielding 1.29 g (5.35 mmol, 36%) of 2*S*-8*Z*-10*E*-13:OAc as a colorless oil.

^{1}H-NMR (400 MHz, $CDCl_3$) δ [ppm] = 0.93 (t, 3H, *J*=7.4 Hz, 13-H); 1.08 (d, 3H, *J*=6.3 Hz, 1-H); 1.12–1.35 (m, 7H, 3-H_a, and 4-H to 6-H); 1.49–1.61 (m, 1H, 3-H_b); 1.72 (s, 3H, $COCH_3$); 1.96–2.06 (m, 2H, 12-H); 2.10–2.19 (m, 2H, 7-H); 4.92–5.01 (m, 1H, 2-H); 5.30–5.38 (m, 1H, 11-H); 5.60–5.70 (m, 1H, 8-H); 6.07–6.15 (m, 1H, 10-H); 6.39–6.50 (m, 1H, 9-H)

^{13}C-NMR (100.6 MHz, $CDCl_3$) δ [ppm] = 13.91 (q, 13-C); 20.08 (q, 1-C); 20.95 (q, CO**C**H_3); 25.66 (t, 12-C); 26.28 (t, 12-C); 27.98/29.38/29.99 (t, 4-C to 6-C); 36.26 (t, 3-C); 70.70 (d, 2-C); 125.41 (d, 9-C); 129.54 (d, 11-C); 129.82 (d, 10-C); 136.30 (d, 8-C); 169.77 (s, *C*O)

MS (70 eV) m/z [%] = 178 (14); 149 (13); 136 (5); 135 (11); 122 (6); 121 (11); 111 (5); 110 (43); 109 (18); 108 (18); 107 (24); 97 (8); 96 (50); 95 (61); 94 (37); 93 (52); 92 (5); 91 (16); 87 (8); 83 (8); 82 (50); 81 (65); 80 (28); 79 (95); 78 (9); 77 (18); 71 (6); 69 (10); 68 (21); 67 (100); 66 (12); 65 (8); 55 (35); 54 (8); 53 (11); 43 (92); 42 (6); 41 (32); 39 (10). For a plotted spectrum, see Fig. 2B.

(2S)-2-(2-Tetrahydropyranyloxy)hept-6-ene (**VI**) Starting from 10 g (74.1 mmol) 4-bromo-1-butene, dissolved in 10 ml abs. tetrahydrofuran and 2.71 g (111 mmol) freshly cut magnesium turnings, a Grignard reagent was prepared at 60°C. This was added dropwise to a suspension of 4.3 g (74.1 mmol) (2*S*)-2-methyloxiran and 1.41 g (7.41 mmol) copper-I-iodide in 50 ml abs. tetrahydrofuran that was cooled to −78°C. After warming to room temperature, the mixture was stirred for an additional hour. Workup started with the addition of 50 ml ethyl acetate and 100 ml of a saturated aqueous solution of ammonium chloride, separation of the layers, and extraction of the aqueous layer (five times with a 1:1 mixture of hexane and ethyl acetate). The combined organic layers were washed with brine and dried over magnesium sulfate. After removal of the solvent *in vacuo*, the crude product was purified by silica column chromatography (hexane–ethyl acetate 1:1), yielding 8.46 g (74.1 mmol, 100%) of (2*S*)-6-hepten-2-ol which was immediately used for the next step. A solution of 8.46 g (74.1 mmol) of (2*S*)-6-hepten-2-ol and 1.80 g (9.92 mmol) *p*-toluene sulfonic acid monohydrate in 70 ml dichloromethane was cooled to −10°C. Subsequently, a solution of

9.67 g (115 mmol) 3,4-dihydro-2*H*-pyran in 20 ml dichloromethane was added dropwise with the temperature kept below 0°C. Stirring was continued for 24 h at 4°C. Workup started with the addition of 200 ml hexane and 200 ml saturated aqueous solution of sodium bicarbonate, separation of the layers, and extraction of the aqueous layer (4×200 ml of a 4:1-mixture of hexane and ethyl acetate). The combined organic layers were washed with brine and dried over magnesium sulfate. After removal of the solvent *in vacuo*, the product was purified by silica column chromatography (hexane–ethyl acetate 97:3), yielding 8.09 g (40.8 mmol, 55%) of the THP derivative **VI**.

^{1}H-NMR (400 MHz, $CDCl_3$) δ [ppm] = 1.30 (d, 3H, *J*= 6.35 Hz, 1′-H); 1.45–1.70 (m, 8H, 3′-H, 4′-H, 3-H_a, 4-H_a, and 5-H); 1.70–1.95 (m, 1H, 3-H_b); 1.85–1.95 (m, 1H, 4-H_b); 2.08–2.20 (m, 2H, 5′-H); 3.52–3.61 (m, 1H, 6-H_a); 3.75–3.90 (m, 1H, 2′-H); 3.92–4.30 (m, 1H, 6-H_b); 4.68–4.80 (m, 1H, 2-H)

^{13}C-NMR (100.6 MHz, $CDCl_3$) δ [ppm] = 19.87 (t, 4-C); 21.70 (q, 1′-C); 25.32 (t, 4′-C); 25.72 (t, 5-C); 31.36 (t, 3-C); 37.12 (t, 3′-C); 62.95 (t, 6-C); 71.04 (d, 2′-C); 98.79 (d, 2-C); 114.50 (t, 7′-C); 139.08 (d, 6′-C)

MS (70 eV) m/z [%] = 101 (18); 96 (20); 86 (5); 85 (100); 84 (5); 81 (7); 67 (12); 57 (10); 56 (25); 55 (52); 54 (9); 45 (5); 43 (14); 41 (29); 39 (12)

*(5S)-5-(2-tetrahydropyranyloxy)hexan-1-ol (***VII***)* A solution of 8.09 g (40.8 mmol) of **VI** in 130 ml of a 1:1 mixture of dichloromethane and methanol was cooled to −78°C. At this temperature, ozone was bubbled through the solution until it turned slightly bluish (about 1 h). Subsequently, oxygen was bubbled through the solution for 10 min. After the addition of 3.11 g (81.6 mmol) solid sodium borohydride and warming to room temperature, stirring was continued for 12 h. Workup started with the careful addition of 100 ml water, separation of the layers, and extraction of the aqueous layer (9×50 ml ethyl acetate). The combined organic layers were dried over magnesium sulfate, and the solvent was removed *in vacuo*. The crude product was chromatographed on silica (hexane–ethyl acetate 4:1) yielding 5.11 g (25.3 mmol, 66%) of the alcohol **VII** as a colorless oil.

^{1}H-NMR (400 MHz, $CDCl_3$) δ [ppm] = 1.20 (d, 3H, *J*= 6.11 Hz, 6-H); 1.35–1.65 (m, 11H, 2-H to 4-H, 3′-H_a, 4′-H_a, 5′-H, and OH); 1.65–1.75 (m, 1H, 3′-H_b); 1.75–1.90 (m, 1H, 4′-H_b); 3.45–3.55 (m, 1H, 6′-H_a); 3.65 (t, 2H, *J*= 6.35 Hz, 1-H); 3.70–3.80 (m, 1H, 5-H); 3.80–3.95 (m, 1H, 6′-H_b); 4.55–4.77 (m, 1H, 2′-H)

^{13}C-NMR (100.6 MHz, $CDCl_3$) δ [ppm] = 20.10 (t, 4′-C); 21.97 (q, 6-C); 25.88 (t, 3-C); 25.91 (t, 5′-C); 31.16 (t, 2-C); 31.61 (t, 3′-C); 39.45 (t, 4-C); 62.78 (t, 6′-C); 67.90 (t, 1-C); 68.39 (d, 5-C); 99.32 (d, 2′-C)

MS (70 eV) m/z [%] = 101 (45); 86 (5); 85 (100); 84 (10); 83 (51); 67 (14); 57 (12); 56 (15); 55 (39); 45 (7); 43 (16); 42 (9); 41 (28); 39 (9)

*(5S)-5-(2-Tetrahydropyranyloxy)hexyl bromide (***VIII***)* A solution of 7.29 g (27.8 mmol) of triphenyl phosphane and 1.89 g (27.8 mmol) imidazole, in a mixture of 120 ml abs. diethyl ether and 40 ml abs. acetonitrile, was cooled to 0°C. Subsequently, 4.44 g (27.78 mmol) bromine were added slowly until a yellowish suspension was formed, and stirring was continued at 20°C for 2 h. After the addition of 5.11 g (25.3 mmol) (5*S*)-5-(2-tetrahydropyranyloxy)hexan-1-ol, dissolved in 15 ml abs. diethyl ether, the mixture was stirred for another 1 h. Workup started with the addition of 150 ml water, separation of the organic layer, and extraction of the aqueous layer (6×120 ml diethyl ether). The combined organic extracts were washed with brine and dried over magnesium sulfate. After removal of the solvent *in vacuo*, the crude product was purified upon column chromatography over silica (hexane–ethyl acetate 96:4). As a result, 5.07 g (19.1 mmol, 75%) of the target bromide **VIII** were obtained as a colorless oil.

^{1}H-NMR (400 MHz, $CDCl_3$) δ [ppm] = 1.23 (d, 3H, *J*= 6.1 Hz, 1′-H); 1.40–1.60 (m, 8H, 3-H_a, 4-H_a, 5-H, 3′-H, and 4′-H); 1.65–1.75 (m, 1H, 3-H_b); 1.78–1.95 (m, 3H, 4-H_b, and 5′-H); 3.42 (t, 2H, *J*=6.87, 6′-H); 3.45–3.54 (m, 1H, 6-H_a); 3.70–3.85 (m, 1H, 2′-H); 3.85–3.95 (m, 1H, 6-H_b); 4.58–4.72 (m, 1H, 2-H)

^{13}C-NMR (100.6 MHz, $CDCl_3$) δ [ppm] = 20.25 (t, 4-C); 21.98 (q, 1′-C); 24.83 (t, 4′-C); 25.94 (t, 5-C); 31.60 (t. 5′-C); 31.64 (t, 3-C); 34.18 (t, 6′-C); 36.01 (t, 3′-C); 63.04 (t, 6-C); 71.23 (d, 2′-C); 99.20 (d, 2-C)

MS (70 eV) m/z [%] = 165 (12); 163 (12); 129 (8); 101 (20); 86 (6); 85 (100); 84 (5); 83 (33); 67 (8); 57 (11); 56 (31); 55 (30); 45 (6); 43 (14); 41 (23); 39 (6)

2S-8E-10E-13:OAc Starting from a mixture of 440 mg (18.1 mmol) freshly crushed magnesium turnings and 40 ml abs. tetrahydrofuran, a Grignard reagent was prepared at 60°C using 2.93 (14.8 mmol) of the bromide **VIII**, dissolved in 10 ml abs. tetrahydrofuran. At −20°C, this solution was added dropwise to a cold solution of 1.52 g (9.87 mmol) (2*E*,4*E*)-2,4-heptadienyl acetate and 3.95 ml (396 mmol) of a 1-M solution of lithium tetrachlorocuprate in tetrahydrofuran. After the addition was complete, stirring

was continued for 5 h at 0°C. Workup started with the addition of 100 ml diethyl ether and 60 ml of a saturated aqueous solution of ammonium chloride, separation of the organic layer, and extraction of the organic layer (5×40 ml diethyl ether). The combined organic solutions were washed with brine and dried over magnesium sulfate. The crude product was purified by silica–10% silver nitrate column chromatography (Ikan 1982) and hexane–ethyl acetate 98:2 as the eluent. A final distillation at 10 Torr per 117°C yielded 1.40 g (4.99 mmol, 51%) of the protected dienol **IX**.

^{1}H-NMR (400 MHz, $CDCl_3$) δ [ppm] = 1.00 (t, 3H, *J*=7.27 Hz, 13′-H); 1.20 (d, 3H, *J*=6.27 Hz, 1′-H); 1.25–1.45 (m, 6H, 4′-H to 6′-H); 1–45–1.65 (m, 6H, 3-H_a, 4-H_a, 5-H, and 3′-H); 1.65–1.75 (m, 1H, 3-H_b); 1.75–1.90 (m, 1H, 4-H_b); 2.00–2.13 (m, 4H, 7′-H, and 12′-H); 3.45–3.52 (m, 1H, 6-H_a); 3.65–3.81 (m, 1H, 2′-H); 3.85–3.95 (m, 1H, 6-H_b); 4.60–4.75 (m, 1H, 2-H); 5.50–5.70 (m, 2H, 8′-H, and 11′-H); 5.95–6.0 (m, 1H, 9′-H); 6.0–6.05 (m, 1H, 10′-H)

^{13}C-NMR (100.6 MHz, $CDCl_3$) δ [ppm] = 14.03 (q, 13′-C); 20.15 (t, 4-C); 21.96 (q, 1′-C); 25.71 (t, 12′-C); 25.97 (t, 5-C); 26.11 (t, 6′-C); 29.67 (t, 4′-C); 29.78 (t, 5′-C); 31.65 (t, 3-C); 32.94 (t, 7′-C); 36.85 (t, 3′-C); 62.82 (t, 6-C); 71.51 (d, 2′-C); 99.01 (d, 2-C); 129.80 (d, 9′-C); 130.80 (d, 10′-C); 132.79 (d, 8′-C); 134.31 (d, 11′-C)

MS (70 eV) m/z [%] = 109 (13); 101 (5); 97 (5); 96 (13); 95 (32); 93 (7); 86 (8); 85 (100); 83 (5); 82 (8); 81 (11); 79 (13); 69 (7); 67 (30); 57 (9); 56 (7); 55 (16); 43 (10); 41 (19)

Deprotection was carried out by stirring a solution of 1.40 g (4.99 mmol) of **IX** and 290 mg (1.56 mol) *p*-toluene sulfonic acid monohydrate in 100 ml methanol for 30 min at 50°C. Subsequently, 835 mg (10.0 mmol) of sodium bicarbonate were added. The solvent was removed *in vacuo*, and the residue was dissolved in 100 ml diethyl ether. The organic solution was dried over magnesium sulfate and concentrated *in vacuo*. Silica column chromatography (hexane–ethyl acetate 4:1) yielded 900 mg (4.59 mmol, 92%) of (2*S*,8*E*,10*E*)-8,10-tridecadien-2-ol as a colorless oil.

^{1}H-NMR (400 MHz, $CDCl_3$) δ [ppm] = 1.00 (t, 3H, *J*=7.52 Hz, 13-H); 1.18 (d, 3H, *J*=6.02 Hz, 1-H); 1.29–1.50 (m, 9H, 3-H to 6-H and OH); 2.02–2.14 (m, 4H, 7-H, and 12-H); 3.74–3.84 (m, 1H, 2-H); 5.50–5.68 (m, 2H, 8-H, and 11-H); 5.95–6.0 (m, 1H, 9-H); 6.0–6.05 (m, 1H, 10-H)

^{13}C-NMR (100.6 MHz, $CDCl_3$) δ [ppm] = 14.02 (q, 13-C); 23.90 (q, 1-C); 25.55/26.01/29.55/29.76/32.89 (t, 4-C to 7-C and 12-C); 39.69 (t, 3-C); 68.53 (d, 2-C); 129.76 (d, 9-C); 130.84 (d, 10-C); 132.63 (d, 8-C); 134.35 (d, 11-C)

MS (70 eV) m/z [%] = 149 (5); 135 (5); 125 (8); 121 (8); 110 (6); 109 (8); 108 (11); 107 (16); 97 (11); 96 (49); 95 (52); 94 (11); 93 (37); 91 (13); 83 (11); 82 (49); 81 (56); 80 (22); 79 (77); 78 (8); 77 (17); 71 (14); 69 (10); 68 (16); 67 (100); 66 (10); 65 (7); 57 (5); 55 (37); 54 (10); 53 (11); 45 (36); 43 (21); 41 (35); 39 (12)

A solution of 900 mg (4.59 mmol) of (2*S*,8*E*,10*E*)-8,10-tridecadien-2-ol and 10 mg *N*,*N*-dimethyl-4-aminopyridine in 10 ml abs. pyridine was cooled to 0°C. Subsequently, 1.29 g (12.8 mmol) acetic anhydride, dissolved in 10 ml tetrahydrofuran, were added dropwise. After warming to room temperature, the mixture was stirred for 2 h. Workup started with the addition of 80 ml diethyl ether and 150 ml of an ice-cold saturated aqueous solution of sodium bicarbonate, separation of the layers, and extraction of the aqueous layer (5×50 ml diethyl ether). The combined organic solutions were washed with 50 ml of an aqueous saturated solution of copper-II-sulfate and 50 ml brine and dried over magnesium sulfate. After removal of the solvent *in vacuo*, the crude product was chromatographed on silica (hexane–ethyl acetate 97:3), yielding 960 mg (4.03 mmol, 88%) of 2*S*-8*E*-10*E*-13:OAc.

^{1}H-NMR (400 MHz, $CDCl_3$) δ [ppm] = 0.99 (t, 3H, *J*=7.38 Hz, 13-H); 1.20 (d, 3H, *J*=6.36 Hz, 1-H); 1.25–1.40 (m, 6H, 4-H to 6-H); 1.43–1.51 (m, 1H, 3-H_a); 1.52–1.62 (m, 1H, 3-H_b); 2.02 (s, 3H, $COCH_3$); 2.02–2.12 (m, 4H, 7-H, and 12-H); 4.84–4.93 (m, 1H, 2-H); 5.50–5.68 (m, 2H, 8-H, and 11-H); 5.95–5.99 (m, 1H, 9-H); 6.00–6.04 (m, 1H, 10-H)

^{13}C-NMR (100.6 MHz, $CDCl_3$) δ [ppm] = 14.01 (q, 13-C); 20.33 (q, 1-C); 21.75 (q, CO**C**H_3); 25.64/25.95/29.36/29.67/32.84 (t, 4-C to 7-C and 12-C); 36.25 (t, 3-C); 71.40 (d, 2-C); 129.75 (d, 9-C); 130.87 (d, 10-C); 132.53 (d, 8-C); 134.35 (d, 11-C); 171.14 (s, *C*O)

MS (70 eV) m/z [%] = 178 (8); 149 (8); 135 (6); 121 (8); 110 (14); 109 (9); 108 (14); 107 (15); 97 (6); 96 (43); 95 (44); 94 (16); 93 (33); 91 (11); 87 (7); 83 (6); 82 (33); 81 (41); 80 (19); 79 (66), 78 (7); 77 (15); 69 (6); 68 (12); 67 (74); 66 (9), 65 (7); 55 (32); 54 (7); 53 (11), 43 (100); 42 (6); 41 (37); 39 (13). For a plotted spectrum, see Fig. 2C.

Y-tube Bioassay The attractiveness of various synthetic pheromone blends and female gland extract were studied in a glass Y-tube olfactometer (modified from Jönsson et al. 2005). The arms of the Y-tube were 14 cm long, with the stem and inner diameter 12.5 and 2.2 cm, respectively. Charcoal-filtered and humidified air was pumped (Micro pump NMP 30 KNDC, 12 V, KNF Neuberger, Germany)

through Teflon tubes and entered each arm via a 9.5-cm-long glass tube. Experiments were conducted between 09:30 and 11:30 at 25°C and 70% RH. The airflow through each arm of the Y-tube was 500 ml/min (approximately 2 cm/s; BA-4AR, Kytölä, Muurame, Finland). For testing chemical stimuli, the stimulus (in hexane) was applied to a piece of filter paper (1.5×0.5 cm) attached to a steel wire (approximately 1.5 cm long); the solvent was allowed to evaporate, and the filter paper was positioned at the tube center. In bioassays in which virgin calling females were used as a stimulus, females were held in small glass tubes (5 cm long, 2.1 cm o.d.; both ends closed by a fine mesh) and placed inside the distal part of one of the Y-tube arms. During tests, a single male was taken from a rearing cage and placed into a glass tube (5 cm long, 2.1 cm o.d.; a fine mesh on the distal end) that was then immediately placed 5 cm into the stem of the Y-tube. Each male was given 5 min to respond. A male was regarded as a responder if it progressed 7 cm up one of the arms of the Y-tube within 5 min. If a male moved 7 cm up one side arm but then moved downwind and then upwind into the other arm, the first arm was regarded as its choice. Males that did not move into one of the two arms were not included in statistical analyses.

Binary-choice bioassays were performed to evaluate differences in attractiveness of the main pheromone component, 2*S*-10*E*-13:OAc (abbreviated 1), a three-component blend (blend abbreviated 3), consisting of 2*S*-10*E*-13:OAc, 2*S*-10*E*-13:OH, and 2*S*-13:OAc, two different four-component blends, consisting of the compounds in blend 3 and with either 2*S*-8*Z*-10*E*-13:OAc (blend abbreviated 4Z) or 2*S*-8*E*-10*E*-13:OAc (blend abbreviated 4E) added, and two five-component blends, both consisting of all compounds included in the other blends but with 2*S*-8*E*-10*E*-13:OAc in different amounts (low amount of 2*S*-8*E*-10*E*-13:OAc abbreviated 5L, high amount 5H). Synthetic pheromone blends were applied in ratios of 10 ng of the main component, 2*S*-10*E*-13:OAc, and 1 ng of the other components in all blends except for 5L in which 0.1 ng of 2*S*-8*E*-10*E*-13:OAc was used. Synthetic blends were compared with a female pheromone gland extract of comparable concentration (with respect to the main component) as well as to five calling females. A blank–blank (hexane only) treatment was also bioassayed to check for non-odor-mediated directional preferences. A maximum of six consecutive males (or 10-min time limit) was tested before filter papers were changed. Potential non-odor-mediated preferences were eliminated by switching sides of the two stimuli during a bioassay. All glass equipment were cleaned by heating at 320°C for 8 h before tests.

Small-Plot Test A small-plot test was done on a fly-free lawn (grass being 6–10 cm high) in Fargo, ND, USA, using delta traps with sticky inserts (PheroNet, Alnarp, Sweden). Seven blocks were run on separate days (September 6–10 and 12–13, 2006), and each block contained six treatments with randomized positions. The three-component blend (2*S*-10*E*-13:OAc, 2*S*-10*E*-13:OH, and 2*S*-13:OAc) and the five-component blend (2*S*-10*E*-13:OAc, 2*S*-10*E*-13:OH, 2*S*-13:OAc, 2*S*-8*E*-10*E*-13:OAc, and 2*S*-8*Z*-10*E*-13:OAc) were tested on two dispenser materials, polyethylene (PE; PE stoppers, 10 mm i.d., Semadeni, Ostermundigen, Switzerland) and cotton (Dental Rolls No. 3 (cut in half), IVF Hartmann AG, Neuhausen, Switzerland). Compounds were applied to the dispensers in a hexane solution. The ratio between 2*S*-10*E*-13:OAc and the other components was 100:10 µg (see also Fig. 6). Empty cotton and PE dispensers were used as controls. Lures and traps were used only once. Newly emerged adult males were collected between 16:00 and 21:00 the night before the test and placed in cylindrical plastic cages (10-cm diameter, 20 cm high, and with a ceiling of cotton netting material) placed over a pot that contained moist potting soil (for further details, see Harris and Foster 1991). At each time of collection, males were evenly distributed between four to five cages. Traps were placed directly on the grass in a line running south to north at 07:00. Since males were released from point sources (cages), we reduced the likelihood of potential position effects by maintaining a small distance (i.e., 30 cm) between traps. At 07:15, cages were taken outside and placed 60 cm apart in a line parallel to the traps. Cages were placed 3 m east (the prevailing wind was from the west) of the trap line with the opening facing the traps. Males were released when all cages were in position. The following numbers of males were released on the 7 days when tests were run: 30, 50, 30, 30, 70, 45, and 30, respectively. Each day, traps were collected at 18:00, and the number of males in each trap was counted. Temperatures ranged from 7°C when males were released to 23°C when traps were collected. Because temperatures were low and the air humid, males that were not attracted to traps probably survived for more than 1 day. Thus, males caught in a trap could have been from previous days' releases.

Field Test A field test was performed in fields of wheat stubble (10–25 cm high) outside Wichita, KS, USA, 15–26 September 2006. A total of 190 delta traps with sticky inserts (same type as in small-plot test) were used, divided into ten blocks with 19 treatments. Traps within a block were placed 5 m apart, and blocks were separated by 10 m. The traps were placed <1 cm above ground level. Three synthetic pheromone treatments were tested: the main component (2*S*-10*E*-13:OAc), the three-component blend (2*S*-10*E*-13:OAc, 2*S*-10*E*-13:OH, and 2*S*-13:OAc), and the five-component blend (2*S*-10*E*-13:OAc, 2*S*-10*E*-13:OH, 2*S*-13:OAc, 2*S*-8*E*-10*E*-13:OAc, and 2*S*-8*Z*-10*E*-13:OAc).

The ratio between the main component and each of the other components was 10:1. Three different doses of each blend were tested, 1, 10, and 100 μg, based on the main component (see also Fig. 7). All blends were tested on PE and cotton dispensers (same types as in small-plot test; dispenser preparation is also the same). A blank unbaited trap was included as negative control. Treatments were placed randomly within blocks and positions were maintained throughout the experiment. Trap catches were checked daily and sticky inserts changed.

Statistics Y-tube bioassay data were analyzed by *chi-square* tests. The data from the small-plot test and field test did not fulfill the requirements for parametric testing due to unequal variances (*Levene's* test, $P<0.001$); catch proportions within blocks were, thus, analyzed by the *Kruskal–Wallis* test. *Mann–Whitney U* tests were used in pairwise comparisons. The *Bonferroni* method was used to calculate experiment-wise error rate with differences at $P<0.005$ for the small-plot test and $P<0.001$ for the field test, being regarded as significant.

Results

GC–EAD recordings of female extracts showed that male antennae responded to seven components. Comparison of mass spectra and retention times of synthetic compounds with corresponding data of the natural products allowed structure elucidation of six volatile substances. The fact that the two laboratories involved in the investigation used GC columns with slightly different selectivity did not present problems. The known pheromone component 2*S*-10*E*-13:OAc served as an orientation marker and, supported by GC–MS analyses, facilitated structural assignments since the target compounds belonged to the same chemical classes. Structure assignments based on GC–MS analyses in Hamburg were scrutinized and verified by GC–EAD in Alnarp. Compound 2 proved to be 2*S*-13:OAc, compound 3, 2*S*-10*Z*-13:OAc, compound 4, 2*S*-10*E*-13:OAc, and compound 5, 2*S*-10*E*-13:OH (Fig. 1). All four compounds had been identified previously (Foster et al. 1991b; Millar et al. 1991). Compounds 1 and 7 were present in insufficient quantities for MS analysis. In contrast, the mass spectrum of compound 6 (Fig. 2a) had a molecular ion at *m*/*z* 238 and signals at *m*/*z* 178 (M^+-60, i.e., loss of acetic acid) and *m*/*z* 61 (protonated acetic acid), as well as intense signals at *m*/*z* 43 (acetyl fragment), *m*/*z* 79, and *m*/*z* 67 (indicating an unsaturated system). These data suggested that the compound was an acetic acid ester of a doubly unsaturated alcohol with 13 carbon atoms. The diagnostic fragment at *m*/*z* 87 revealed the acetate moiety to keep position 2 along the chain (α-cleavage). The relatively large abundance of the molecular ion (approximately 5–6%) suggested a conjugated double-bond system (Löfstedt and Odham 1984). As the main pheromone component, compound 4 has a double bond in position 10, we assumed that compound 6 was a 2-acetoxytridecadiene with double bonds in positions 8,10 or 10,12. Because of the relatively small difference in retention times between the diene 6 and the monoene 4, the 8,10 system seemed a more likely candidate compared to the terminally unsaturated 10,12 system, which, due to its higher polarity, would be expected to have a much longer retention time. This was supported by the presence of a pronounced signal at *m*/*z* 149, indicating a terminal ethyl group (M+-acetic acid-ethyl). In addition, compound 6 was hypothesized to keep (2*S*)-configuration, as other compounds identified in the Hessian

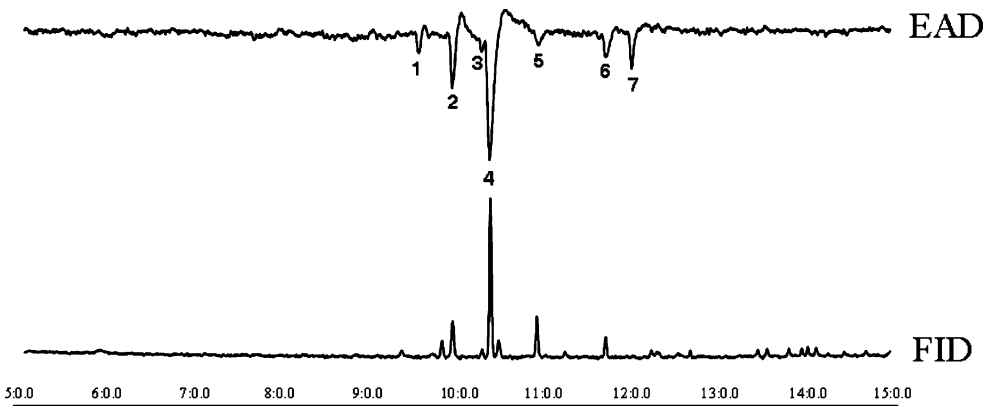

Fig. 1 Coupled flame ionization (*FID*) and electroantennographic detection (*EAD*) of female Hessian fly extract (20 female equivalents). Male antennal responses were recorded to seven compounds: *1* unknown, *2* (2*S*)-tridec-2-yl acetate [2*S*-13:OAc], *3* (2*S*,10*Z*)-10-tridecen-2-yl acetate [2*S*-10*Z*-13:OAc], *4* (2*S*,10*E*)-10-tridecen-2-yl acetate [2*S*-10*E*-13:OAc], *5* (2*S*,10*E*)-10-tridecen-2-ol [2*S*-10*E*-13:OH], *6* (2*S*,8*Z*,10*E*)-8,10-tridecadien-2-yl acetate [2*S*-8*Z*-10*E*-13:OAc], and *7* (2*S*,8*E*,10*E*)-8,10-tridecadien-2-yl acetate [2*S*-8*E*-10*E*-13:OAc]

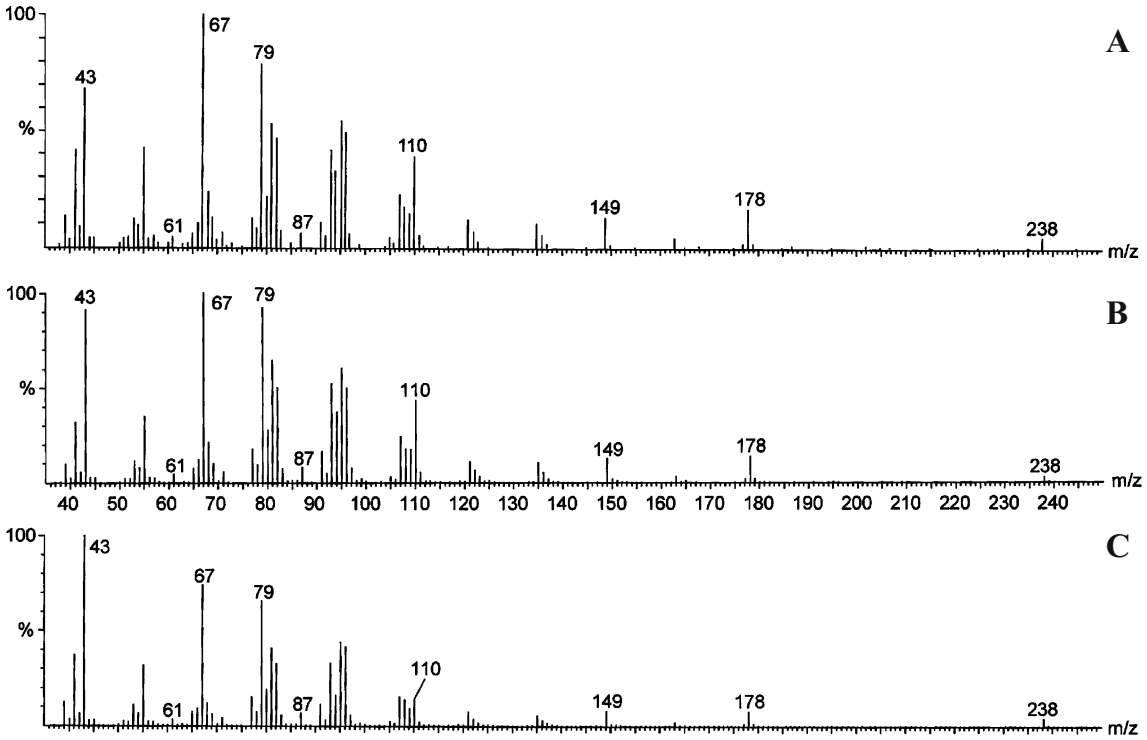

Fig. 2 The 70-eV mass spectra of **a** compound 6 present in Hessian fly females, **b** synthetic (8*Z*,10*E*)-8,10-tridecadien-2-yl acetate, and **c** synthetic (8*E*,10*E*)-8,10-tridecadien-2-yl acetate

fly pheromone gland (Foster et al. 1991b; Millar et al. 1991). Consequently, (2*S*,8*Z*,10*E*)-8,10-tridecadien-2-yl acetate [2*S*-8*Z*-10*E*-13:OAc] and its (8*E*,10*E*)-isomer [2*S*-8*E*-10*E*-13:OAc] were synthesized for comparison.

The preparative routes to the synthetic products are summarized in Figs. 3 and 4. The compounds were obtained in overall yields of approximately 10%. With respect to the conjugated double-bond system, the stereochemical purity of 2*S*-8*Z*-10*E*-13:OAc was 96%, containing approximately 4% of the (*E*,*E*)-isomer while that of 2*S*-8*E*-10*E*-13:OAc was 98% and contained approximately 2% of its stereoisomers. Enantioselective gas chromatography proved 2*S*-8*Z*-10*E*-13:OAc to show an excess of the (*S*)-enantiomer over the (*R*)-enantiomer of at least 98%, while 2*S*-8*E*-10*E*-13:OAc showed an enantiomeric excess of 96%.

The mass spectra of the two synthetic compounds were very similar. However, a significant difference in the intensity of the signal at *m*/*z* 110 was found. The mass spectrum and retention time of synthetic 2*S*-8*Z*-10*E*-13:OAc matched perfectly the corresponding data of the natural compound 6. In addition, the synthetic compound elicited behavioral (see below) and electrophysiological activities. A small impurity in the synthetic sample of 2*S*-8*Z*-10*E*-13:OAc (regarded as the 8*E*,10*E*-isomer, an expected by-product of the Wittig reaction) matched the retention time of compound 7. Because the concentration of this compound was very low, a mass spectrum of the natural product could not be obtained. However, by using an Innowax column, the GC retention time of synthetic 2*S*-8*E*-10*E*-13:OAc corresponded precisely to the retention

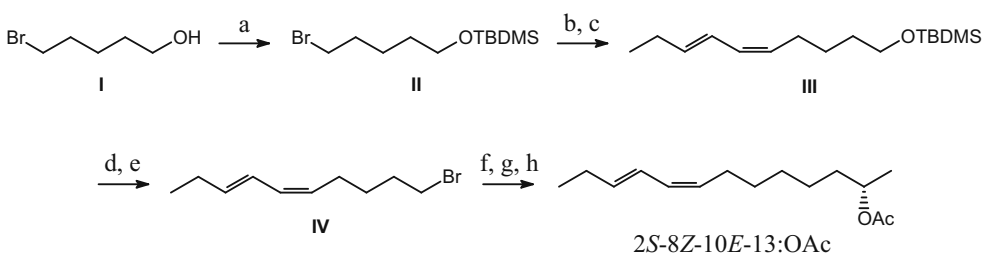

Fig. 3 Synthetic route to 2*S*-8*Z*-10*E*-13:OAc. *a*: TBDMSCl, imidazole, 0°C, DMF; *b*: PPh_3, Na_2CO_3, reflux, CH_3CN; *c*: (2*E*)-2-pentenal, KHMDS, 0°C, THF; *d*: $Bu_4N^+F^-$, 20°C, THF; *e*: PPh_3, Br_2, imidazole, 0°C, DCM; *f*: Mg, −78°C, CuI, THF; *g*: (2*S*)-2-methyloxiran, −78°C; *h*: Ac_2O, DMAP, 0°C, pyr

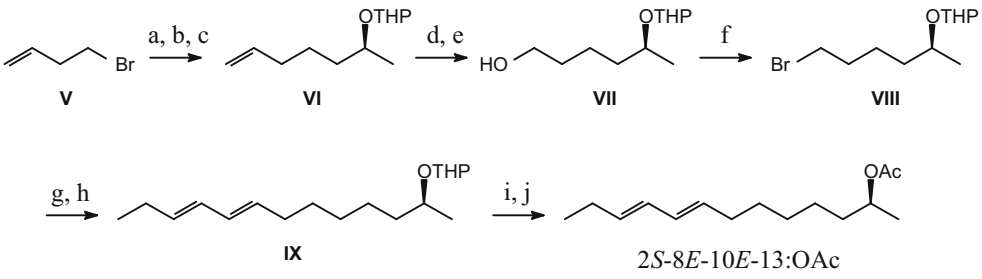

Fig. 4 Synthetic route to 2*S*-8*E*-10*E*-13:OAc. *a*: Mg, −78°C, CuI, THF; *b*: (2*S*)-2-methyloxiran, −78°C; *c*: *p*-TsOH, DHP, DCM; *d*: O_3, −80°C, DCM/MeOH; *e*: $NaBH_4$, 20°C. *f*: PPh_3, Br_2, imidazole, 0°C, DCM; *g*: Mg, 60°C, Li_2CuCl_4, THF; *h*: (2*E*,4*E*)-2,4-heptadienyl acetate; *i*: *p*-TsOH, 60°C, MeOH; *j*: Ac_2O, DMAP, 0°C, pyr

time of the compound that elicited antennal response number 7 (Fig. 1). As GC–EAD recordings were not carried out on an enantioselective GC column, the enantiomeric composition of the tentatively identified tridecadien-2-yl acetate remains unknown. However, the synthetic (*S*)-enantiomer elicited behavioral responses from males (see below) as well as electrophysiological activity. We, therefore, postulate 2*S*-*E*8-*E*10-13:OAc to be the natural substance causing response 7 in Fig. 1.

Y-tube Bioassay The proportion of males responding was high in all bioassays (86–100%), except in the blank–blank treatment (53%). The blank–blank bioassay demonstrated that males did not have non-odor-mediated directional preferences; ten of the responding males chose the right arm and 11 chose the left arm (χ^2=0.048, P>0.05). When a blank was tested against the main pheromone compound 2*S*-10*E*-13:OAc (attractant 1), males were more attracted to the latter component (χ^2=33.92, P<0.001; Fig. 5). However, when 2*S*-13:OAc and 2*S*-10*E*-13:OH were added to the main component, males were more attracted to the three-component blend (attractant 3) than to the main component alone (1; χ^2=10.29, P<0.01). A tendency for increasing numbers of males responding to the blend was observed when either 2*S*-8*E*-10*E*-13:OAc or 2*S*-8*Z*-10*E*-13:OAc was added to the three-component blend (attractants 4E and 4Z, respectively), but these blends did not attract significantly more males than blend 3 (3 vs. 4E: χ^2=1.72, P>0.05; 3 vs. 4Z: χ^2=1.53, P>0.05). There was also no difference between the responses to 3 and the five-component blend with the low amount (0.1 ng) of 2*S*-8*E*-10*E*-13:OAc (attractant 5L; χ^2=0.022, P>0.05). However, when 2*S*-8*E*-10*E*-13:OAc was present in the same amount (1 ng) as the other minor components (attractant 5H), the five-component blend attracted more males than the three-component blend (3; χ^2=8.40, P<0.01). 4Z and 5H were both compared with gland extract. The results from these comparisons showed that gland extract was more attractive than the four-component blend (4Z; χ^2=17.47, P<0.001) but that there was no difference between the gland extract and the five-component blend (5H; χ^2=1.28, P>0.05). The responses of males to 5H or five calling females (5FE) did not differ significantly either (χ^2=2.00, P>0.05).

Small-Plot Test During the 7 days of testing, a total of 285 males were released; 218 were caught in the traps. Traps containing the five-component blend caught more male Hessian flies than blank traps (Fig. 6; Z=−3.343, P<0.001 for both dispenser types) or the three-component blend (Z=−3.144, P<0.001 for both dispenser types). Traps baited with the three-component blend did not catch significantly more males than blank traps. The attractiveness of the five-component blend did not differ between dispenser types.

Field Test During the 9 days of field testing, a total of 963 male Hessian flies were caught. Of these, only eight individuals were caught in the cotton dispenser traps. Therefore, we excluded results from traps with this type of dispenser from further analysis. In traps with PE dispensers, a total of 848 males were caught at the largest dose (100 µg) of the five-component blend (blend 9, Fig. 7), while 93 males were caught in traps containing the same blend, but at the lower dose (10 µg, blend 8, Fig. 7). Mean trap catches of these treatments differed significantly from catches in the blank (Z=−3.963, P<0.001 for both doses) and from each other (Z=−3.780, P<0.001; Fig. 7). To ensure the specificity of this system, sticky inserts with trapped midges were characterized for species and sex; all midges caught were Hessian fly males (R. Gagné, personal communication). Reference males are stored in the North Dakota State University insect collection (reference number 6543, lot # 0610677).

Discussion

We have shown that a synthetic blend of five out of seven electrophysiologically active compounds found in Hessian fly female sex pheromone gland extract was highly attractive to laboratory-reared males and to males from natural populations. A sixth compound, the *Z*-isomer of the main pheromone component, 2*S*-10*E*-13:OAc, was not

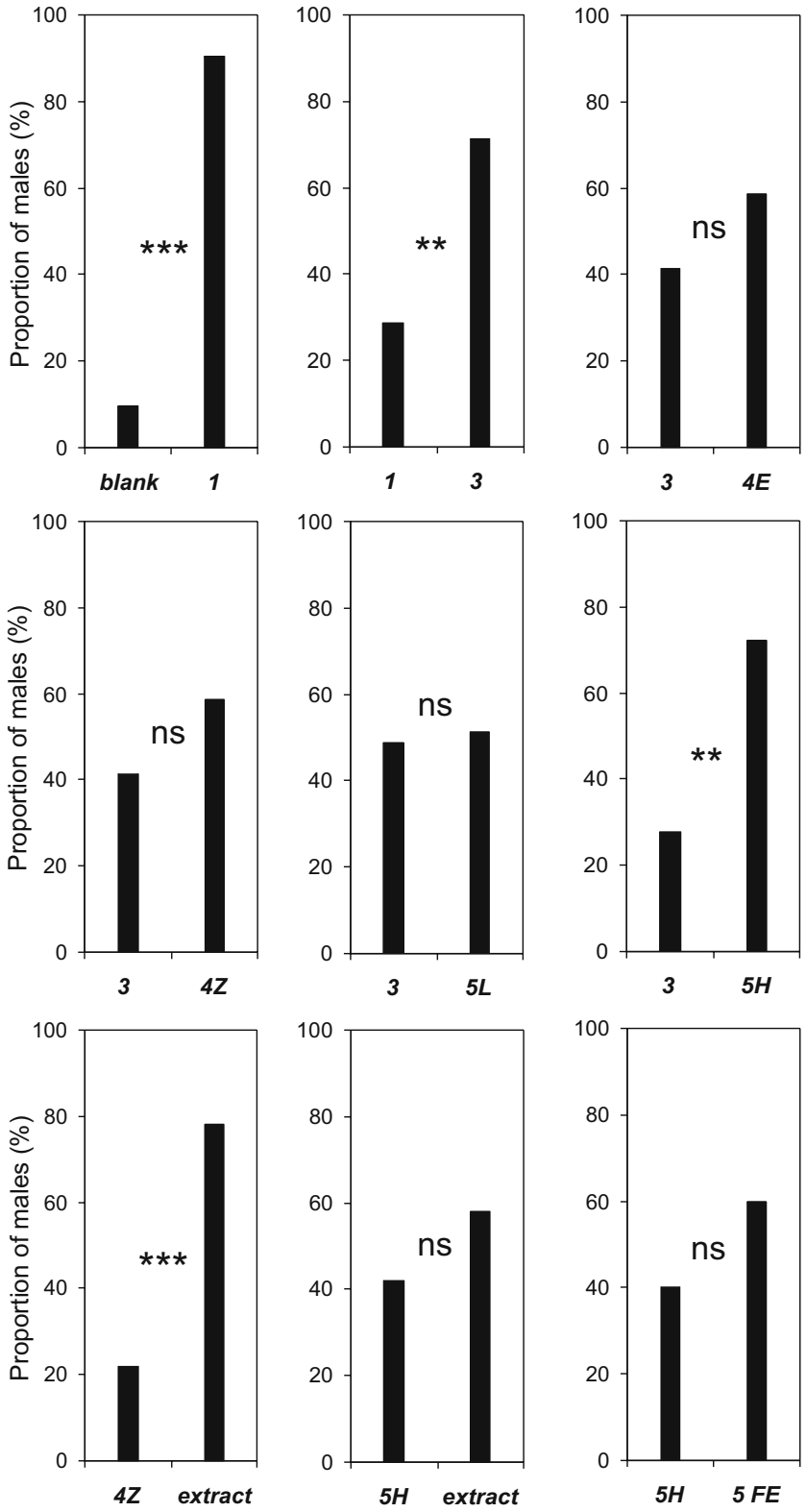

Fig. 5 Responses of male Hessian flies in binary-choice bioassays. Abbreviations: *1*: 2*S*-10*E*-13:OAc (10 ng), *3*: 2*S*-10*E*-13:OAc, 2*S*-13:OAc, and 2*S*-10*E*-13:OH (10:1:1 ng), *4E*: 2*S*-10*E*-13:OAc, 2*S*-13:OAc, 2*S*-10*E*-13:OH, and 2*S*-8*E*-10*E*-13:OAc (10:1:1:1 ng), *4Z*: 2*S*-10*E*-13:OAc, 2*S*-13:OAc, 2*S*-10*E*-13:OH, and 2*S*-8*Z*-10*E*-13:OAc (10:1:1:1 ng), *5L*: 2*S*-10*E*-13:OAc, 2*S*-13:OAc, 2*S*-10*E*-13:OH, 2*S*-8*Z*-10*E*-13:OAc, and 2*S*-8*E*-10*E*-13:OAc (10:1:1:1:0.1 ng), *5H*: as *5L* but (10:1:1:1:1 ng) and *5FE:* five calling females. *N*=40–60, * = $P<0.05$, ** = $P<0.01$, and *** = $P<0.001$

included in the blend as a separate synthetic chemical. However, due to the isomeric purity of the synthetic 2*S*-10*E*-13:OAc (98%), a small amount of the *Z*-isomer was present in the blend. A low ratio of the *Z*-isomer was also found in female extract (Millar et al. 1991). Since an earlier wind tunnel study had demonstrated that different *E*/*Z* ratios of the main component did not influence male attraction (Harris and Foster 1991), no other ratios were tested.

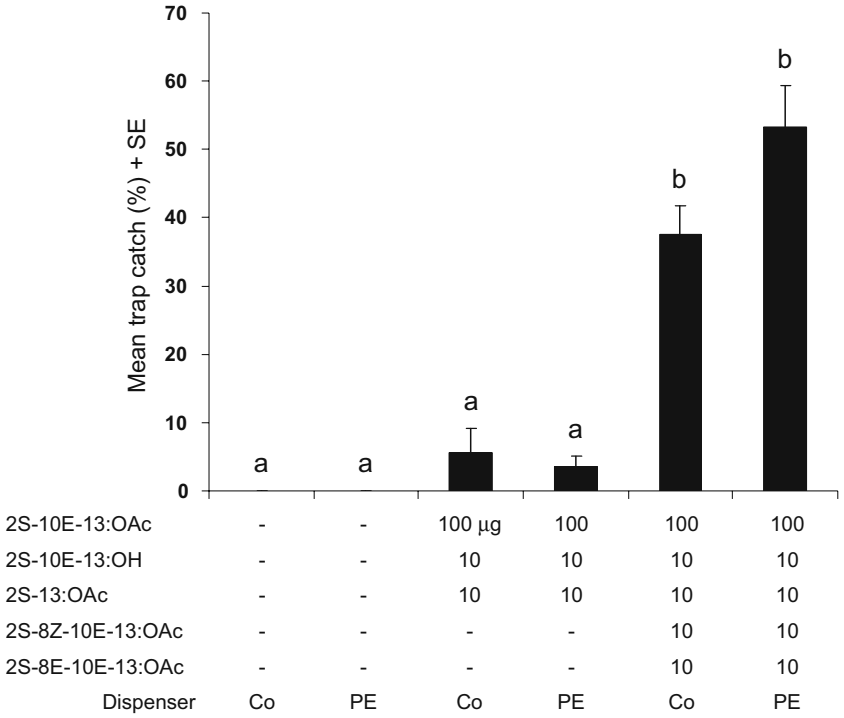

Fig. 6 Mean male Hessian fly trap catch (%)+SE in the small-plot test. *Numbers* represent microgram amounts per compound. *Bars* with different *letters* are significant at $P<0.001$. *Co* = cotton, *PE* = polyethylene

The five-component blend was as active as female extract in the Y-tube bioassays and sufficiently active for monitoring field populations of Hessian fly. Since the highest catch was in traps with the highest dose, we do not know whether we reached the optimal dose for attraction by this blend. However, in pheromone-based monitoring systems, it is desirable to reach sufficient attraction at low doses. For this reason, it would be of interest to identify the seventh EAD-active compound (response 1 in Fig. 1), as nothing is known regarding its capacity to mediate behavior. Studies of various moth species have shown that addition of minor components lowers the behavioral response threshold

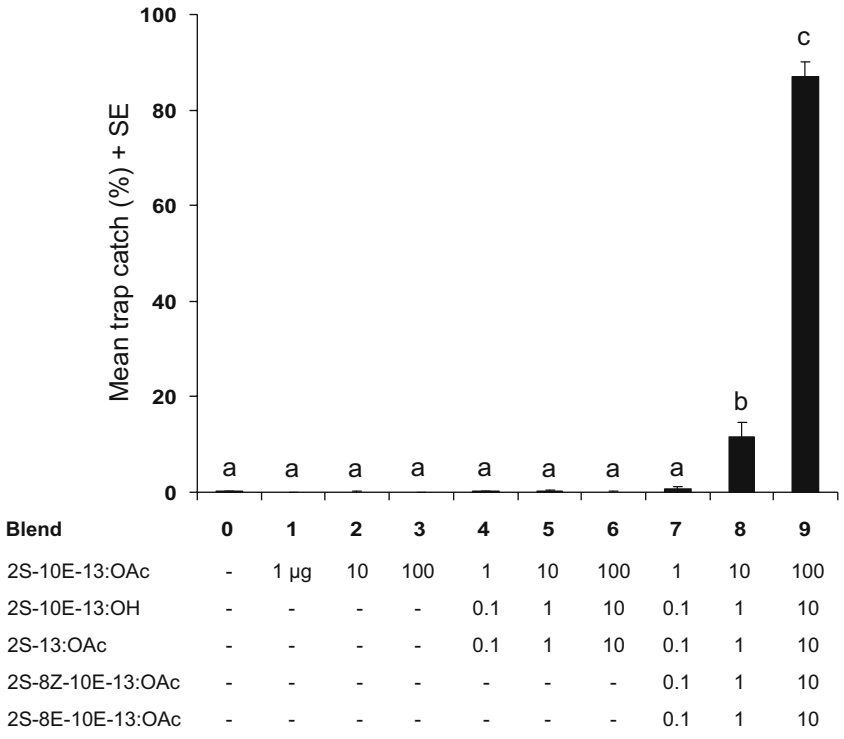

Fig. 7 Mean male Hessian fly trap catch (%)+SE in traps with polyethylene dispensers in the field test. *Numbers* represent microgram amounts per compound. Catch in blank traps is also shown. *Bars* with different *letters* are significant at $P<0.001$

to the pheromone blend (reviewed by Linn and Roelofs 1995), and it is possible that attractiveness to pheromone traps could be achieved at a lower dose, should this component be added. However, this minor compound is present in very low levels in gland extracts, as is the compound corresponding to response 7 (see Fig. 1) tentatively identified as 2*S*-8*E*-10*E*-13:OAc. Despite the minute amounts of 2*S*-8*E*-10*E*-13:OAc in the extract, the Y-tube experiment indicates that this compound must be present in higher amounts (the same amount as for the other minor compounds) in order to increase the attractiveness of the pheromone blend. The ratios of compounds found in the gland extract might thus not be reflective of the compound ratios released by calling females, suggesting that collection of volatiles released from females might allow the collection of greater quantities of this compound and facilitate structure confirmation. This may also apply to the unknown compound associated with response 1 (Fig. 1).

The structures of Hessian fly sex pheromone components are similar to other gall midge pheromones in that they are chiral compounds with odd-numbered carbon chains and a functional group at the C-2 position (Foster et al. 1991b; Harris and Foster 1991; Gries et al. 2000, 2002, 2005; Hillbur et al. 1999, 2000, 2001, 2005; Choi et al. 2004). In contrast, the attractive sex pheromone blends from other cecidomyiids are composed of either one (Gries et al. 2000, 2002; Choi et al. 2004) or three compounds (Hillbur et al. 1999, 2005; Gries et al. 2005), compared to the complex Hessian fly blend. In the laboratory, the main Hessian fly pheromone component attracted 56% of males when tested in a wind tunnel (Harris and Foster 1991). In our study, more than 90% of the responding males were attracted to this component when tested against a blank in the Y-tube. When tested against more complex blends, the main component alone was not attractive. The attractiveness of the main Hessian fly sex pheromone component probably is reflective of the different challenges faced by male flies when orienting to the pheromone in highly controlled laboratory conditions vs. variable field conditions. In the field, synthetic pheromone lures presumably compete with calling females. The presence of calling females around traps in the field might have contributed to the low attractiveness of the main component and the three-component blend. The only discrepancy between the trap catches in the small-plot test and in the field test was that cotton dispensers were as attractive as PE dispensers in the small-plot test. Chemicals are typically released from cotton dispensers at a faster rate than from polyethylene ones. In the small-plot test, we were able to change the dispensers daily but did not do so in the field trial. This may explain the differences between the two types of tests.

The sex pheromone blend that was identified in this study could be used as a tool for detecting the presence and abundance of Hessian flies in the field. The Hessian fly is a major pest of winter wheat. A common approach to reduce infestation levels in the field is to delay crop planting until Hessian fly activity has ceased in autumn (Buntin et al. 1990). Since flight activity is highly variable from year to year, a pheromone-based detection method could enable farmers to better judge when Hessian fly activity is over for the season. The traps that were used in the small-plot test and field assay were placed at (or almost at) ground level. The fact that male Hessian flies were caught at this height suggests that they fly close to the ground, at least for within-field dispersal. This has also been demonstrated for both the swede midge (Hillbur et al. 2005) and the pea midge (Wall et al. 1991).

An important characteristic of a pheromone-based monitoring system is that it is specific for the target species and does not catch insects that are morphologically similar. In our field trial, the only midges caught in traps baited with our synthetic pheromone blend were Hessian fly males. With this specificity and the highly synchronized emergence of Hessian fly adults (Harris and Foster 1999), it appears that the five-component blend may be a useful tool for monitoring and predicting Hessian fly outbreaks in crops.

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Male-Produced Aggregation Pheromone of the Cerambycid Beetle *Rosalia funebris*

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Abstract We report the identification, synthesis, and field bioassays of a volatile, male-produced aggregation pheromone of a long-horned beetle, the banded alder borer, *Rosalia funebris* Mots. Headspace collections from males contained a major male-specific compound, (*Z*)-3-decenyl (*E*)-2-hexenoate, and several minor components, identified as (*Z*)-3-decenol, (*Z*)-3-nonenyl (*E*)-2-hexenoate, and (*Z*)-3-decenyl (*E*)-3-hexenoate. The antennae of both males and females responded strongly to (*Z*)-3-decenyl (*E*)-2-hexenoate. We collected significant numbers of adult *R. funebris* in field bioassays using traps baited with this compound. This pheromone structure is unprecedented in the literature of cerambycid pheromones and distinct from the more common diol/hydroxyketone pheromone motif of many other species of the diverse subfamily Cerambycinae. This is the first pheromone identified for a species in the tribe Rosaliini.

Keywords Aggregation pheromone · Sex pheromone · (*Z*)-3-decenyl (*E*)-2-hexenoate · Cerambycidae · Banded alder borer · Long-horned beetle · Woodborer

Introduction

To date, male-produced sex or aggregation pheromones have been reported for 11 species in three tribes of the cerambycid subfamily Cerambycinae (reviewed by Ray et al. 2006; Hanks et al. 2007; Lacey et al. 2008b). Pheromones of most of these species are comprised of one to four compounds that are 6, 8, or 10 carbons in length and that share a common structural motif of hydroxyl or carbonyl groups at C_2 and C_3 (Hanks et al. 2007; Lacey et al. 2008b). Exceptions include *Phymatodes lecontei* Linsley, males of which appear to produce only (*R*)-2-methylbutan-1-ol (Hanks et al. 2007), and *Megacyllene caryae* (Gahan), in which males produce a pheromone blend comprised of 2,3-hexanediols, terpenoids, and an aromatic alcohol (Lacey et al. 2008b). Pheromones of male cerambycines of many species are emitted through male-specific gland pores in the prothorax (Ray et al. 2006; Hanks et al. 2007). In some cerambycine species, however, prothoracic gland pores are absent in both sexes, suggesting that adults either do not produce volatile pheromones or produce them from other sources (Ray et al. 2006).

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Here, we report the identification, synthesis, and field bioassays of the male-produced aggregation pheromone of a cerambycine species, the banded alder borer *Rosalia funebris* Mots., that lacks male-specific prothoracic pores (Ray et al. 2006). This attractive black-and-white patterned species is endemic to western North America (Linsley 1964) and, in fact, was one of the first insects described from this region (Linsley 1995). Larvae of *R. funebris* develop in woody tissues of trees in the genera *Alnus*, *Fraxinus*, *Quercus*, *Salix*, and *Umbellularia* (Linsley 1964). Adults emerge from April to August (depending on latitude, elevation, and ambient temperature) and mate. Females oviposit on trees that have recently died (Linsley 1964, 1995). The adults are diurnal but rarely encountered in their natural habitat. Consequently, little is known of their biology and behavior (Essig 1943; Linsley 1964, 1995). Both sexes occasionally aggregate on hot days on buildings that have been recently painted, and these aggregations have been the subject of many reports in the popular press (Chemsak and Linsley 1971; Linsley 1995). In the work reported here, we provide evidence that (*Z*)-3-decenyl (*E*)-2-hexenoate is the major component of the male-produced aggregation pheromone of *R. funebris*.

Materials and Methods

Identification of Pheromone Components Adult *R. funebris* of both sexes (mating status unknown) were collected on 24–27 June 2007 from an aggregation on a building that was painted shortly prior to this period (Chipotle Mexican Grill, 3409 E. Foothill Blvd., Pasadena, CA, USA; 34°9′1″ N, 118°04′49″ W, 218 m elevation). Headspace volatiles were collected from six females and five males, held individually in either 3 cm diameter×20 cm long, glass aeration chambers or 263-ml glass canning jars with the screw-cap metal lids modified to accommodate air inlet and outlet tubes. Beetles in chambers were provided with water in 2-ml glass vials plugged with cotton wicks. Air entering the chambers was purified by passage through activated charcoal with the volatile chemicals trapped on a 5-mm plug of thermally desorbed activated charcoal (50–200 mesh) held in place by glass wool plugs in a section of 7-mm OD glass tubing attached to the chamber outlet. Air was pulled through the chambers at ~300 ml min^{-1} by vacuum. Aerations were conducted in an environmentally controlled room (25°C, 50% room temperature, 16:8 h ratio of light/dark, fluorescent lighting). Aerations were run for 2–7 days, before collectors were extracted with three 200-µl rinses of methylene chloride. The extracts were combined and analyzed as described below. In total, 24 and 33 beetle days of volatiles were collected from males and females, respectively.

Aliquots of crude extracts of volatiles collected from both sexes were analyzed by gas chromatography–electroantennogram detection (GC–EAD) with custom-built equipment as described in McElfresh and Millar (1999), using antennal sections from three male and three female *R. funebris* captured in 2007 (see above). Antennal preparations consisted of the terminal 1–2 cm of a beetle antenna, with the tip cut off with a razor blade, suspended between two saline-filled glass electrodes. Extracts were analyzed with Hewlett-Packard 5890A or 5890 series II GCs (Hewlett-Packard, Palo Alto, CA, USA) with helium carrier gas, splitless injection at 250°C, and a DB-5 column (30 m×0.25 mm ID, 0.25 µm film; J&W Scientific, Folsom, CA, USA) programmed from 40°C (held for 1 min) to 275°C at 10°C min^{-1}. We confirmed that synthetic (*Z*)-3-decenyl (*E*)-2-hexenoate elicited strong responses from antennae of beetles of both sexes by GC–EAD, using beetles collected during field bioassays in 2008 (see below).

Electron impact mass spectra (EIMS; 70 eV) were recorded with an HP 6890 gas chromatograph interfaced to an HP 5973 mass selective detector (GC–MS). An HP5-MS column (30 m×0.25 mm ID) was used in splitless mode with helium carrier gas, programmed from 40°C (held for 1 min) to 250°C at 10°C min^{-1}, with injector and transfer line temperatures at 275°C and 280°C, respectively. Compounds were identified by comparison of retention times and mass spectra with those of authentic standards. To further verify identifications, extracts and synthetic standards were analyzed on a polar DB-Wax column (30 m×0.25 mm ID×0.25 µm film, J&W Scientific) programmed from 50°C (held for 1 min) to 250°C at 10°C min^{-1}.

Microchemical Reactions on Aeration Extracts Reduction was carried out by adding approximately 1 mg of 5% Pd on carbon to a 1.5-ml vial containing 100 µl of a crude aeration extract from a male beetle. The vial was flushed with H_2 and sealed and held under H_2 atmosphere while stirring for 1 h. The mixture was then filtered through a plug of Celite® to remove the catalyst and analyzed by GC–MS.

Positions of double bonds were determined by epoxidation of an aliquot of the extract (Hogge and Millar 1987). Thus, an aliquot was treated with two drops of a CH_2Cl_2 solution of *m*-chloroperbenzoic acid (5 mg/ml), and the mixture held at room temperature for 1 h, swirling periodically. The mixture was diluted with 1 ml pentane and extracted twice with 0.5 M aqueous NaOH. The pentane layer was dried over Na_2SO_4, concentrated under a stream of N_2, and analyzed by GC–MS as described above. Samples of synthetic (*Z*)-3-decenol and (*Z*)-3-decenyl (*E*)-2-hexenoate were submitted to the same procedure to provide standards for comparison with aeration extracts.

Chemicals Decyl hexanoate and (*Z*)-4-decenol were available from our in-house library of insect semiochemicals. (*Z*)-3-Decenol and (*Z*)-3-decenyl (*E*)-2-hexenoate were synthesized as follows:

(Z)-3-Decenol This compound was prepared by P-2 nickel reduction of 3-decyn-1-ol (Brown and Ahuja 1973). Ni $(OAc)_2 \cdot 4H_2O$ (1.5 g, 6 mmol) was dissolved in 120 ml EtOH, and the solution degassed by bubbling N_2 for ~15 min. Then, still under N_2 atmosphere, 1 M $NaBH_4$ solution (6 ml of a solution prepared from 0.4 g $NaBH_4$, 9.5 ml EtOH, and 0.5 ml 1 M NaOH, filtered to remove suspended material) was added by syringe, with vigorous stirring. Ethylene diamine (2.4 ml) was added to the resulting black solution, the flask was fitted with a balloon filled with H_2, the N_2 line was removed, and the solution was stirred 15 min before adding 3-decyn-1-ol (10 g, 64 mmol, Alfa-Aesar, Ward Hill, MA, USA) dropwise over 10 min. The mixture was stirred until the starting material had been consumed completely (determined by GC, ~1.5 h). The flask then was flushed with N_2, and the mixture was filtered through a plug of Celite® (~2 cm) and activated charcoal (~1 cm top layer). The resulting violet colored solution was concentrated, taken up in ether, and extracted with 1 M HCl. The ether layer was washed with saturated aqueous $NaHCO_3$ and brine, concentrated, and Kugelrohr distilled (bp ~70°C, 0.2 mm Hg), giving a quantitative yield of the alcohol as a colorless oil EIMS (70 eV); *m/z* (percent abundance): 156 (1), 138 (12), 110 (20), 95 (59), 81 (81), 67 (100), 55 (75), and 41 (51); ^{1}H nuclear magnetic resonance (NMR; 400 MHz, $CDCl_3$): δ 5.55 (m, 1H, H-3), 5.34 (m, 1H, H-4), 3.62 (t, 2H, *J*= 7.4 Hz, H-1), 2.31 (br overlapped dt, 2H, H-2), 2.04 (br overlapped dt, 2H, H-5), 1.47 (br s, 1H, OH), 1.4–1.2 (m, 8H, H6–H9), and 0.86 (t, 3H, *J*=7.0 Hz, H-10); and ^{13}C NMR: δ 133.8, 125.1, 62.5, 31.9, 31.0, 29.9, 29.2, 27.6, 22.9, 14.3].

(Z)-3-Decenyl (E)-2-hexenoate A mixture of (*E*)-2-hexenoic acid (6.84 g, 60 mmol; Acros Organics, Morris Plains, NJ, USA), (*Z*)-3-decenol (6.2 g, 40 mmol), and *p*-toluenesulfonic acid (0.5 g) was refluxed overnight in 50 ml benzene, with a Dean-Stark trap to remove water. After cooling to room temperature, 100 ml of saturated aqueous $NaHCO_3$ were added, and the mixture was stirred 10 min. The layers were separated, and the organic layer was washed with aqueous $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4, and concentrated. The residue was purified by Kugelrohr distillation (oven temperature ~105–120°C, 0.05 mm Hg), yielding 9.5 g of the ester as a colorless oil (94%, purity >96% by GC) EIMS (70 eV); *m/z* (percent abundance) 252 (trace), 138 (70), 115 (12), 110 (42), 97 (95), 81 (55), 68 (81), 55 (100), and 41 (45); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.94 (dt, 1H, *J*=15.6, 7 Hz, H-3′), 5.79 (d, 1H, *J*=15.6 Hz, H-2′), 5.48 (m, 1H, H-3), 5.35 (m, 1H, H-4), 4.11 (t, 2H, *J*= 6.6 Hz, H-1), 2.38 (br overlapped dt, 2H, *J*=~7, 7 Hz, H-2), 2.16 (overlapped dtd, 2H, *J*=~7, 7, 1.6 Hz, H-4′), 2.02 (br overlapped dt, 2H, *J*=~7 Hz, H-5), 1.47 (overlapped tq, 2H, H-5′), 1.4–1.2 (m, 8H, H6-H9), 0.92 (t, 3H, *J*=7.6 Hz, H6′), and 0.87 (t, 3H, *J*=6.8 Hz, H-10); and ^{13}C NMR δ 167.0, 149.5, 133.2, 124.6, 121.5, 63.9, 34.4, 32.0, 29.8, 29.2, 27.5, 27.1, 22.8, 21.5, 14.3, and 13.9].

Authentic samples of (*Z*)-3-nonenyl (*E*)-2-hexenoate and (*Z*)-3-decenyl (*E*)-3-hexenoate, for confirming the identities of the minor components, were synthesized by analogous esterification reactions between (*Z*)-3-nonenol (TCI America, Portland, OR, USA) and (*E*)-2-hexenoic acid and (*Z*)-3-decenol and (*E*)-3-hexenoic acid (Aldrich Chem. Co., Milwaukee, WI, USA).

Field Bioassays of Synthetic Compounds The responses of adult *R. funebris* to the major component of the male aeration, (*Z*)-3-decenyl (*E*)-2-hexenoate, were tested in field bioassays at Placerita Canyon Natural Area (Los Angeles Co., CA, USA). Black flight-intercept panel traps (1.2 high×0.3 m wide, model PT Intercept™, APTIV, Portland, OR) were used in the trials. Traps were modified by replacing the collection basin with a 1.9-l plastic funnel that was attached to a clear plastic jar (1.9 l, General Bottle Supply Company, Los Angeles, CA, USA), in order to capture beetles alive. The funnel spout was cut off to leave a 3.5-cm-diameter hole at the bottom, a ~10-cm hole was cut into the threaded lid of the jar, and the funnel spout was hot-melt glued to the lid so that the spout was inside the jar. Traps were hung from tree branches ~2 m above the ground. Lures consisted of clear low-density polyethylene press-seal bags (Bagette model 14770, 5.1×7.6 cm, 0.05 mm wall thickness, Cousin Corp., Largo, FL, USA). The plastic bags were sealed and suspended with wire in the central open area of traps.

A sentinel trap baited with 100 μl of neat (*Z*)-3-decenyl (*E*)-2-hexenoate was used to monitor adult *R. funebris* at the Placerita Canyon Natural Area. The trap was set up on 18 June 2008, based on a report of an adult *R. funebris* observed in the Natural Area on that date in 2007 (AMR, unpublished data). The field bioassay was started on the day that the first adult was captured, 22 June 2008, and continued daily through 3 July 2008 (clear skies, no precipitation, maximum air temperatures 28–42°C). Traps were hung from tree branches, with traps separated by ~10 m, in a northeasterly transect through a canyon containing mature California sycamore, *Platanus racemosa* Nutt., and coast live oak, *Quercus agrifolia* Neé (position of first trap: 34°22′ 39″ N, 118° 28′ 1″ W, 466 m elevation). Many trees in the area had been burned by recent wildfires. We tested the responses of beetles to two concentrations of

(*Z*)-3-decenyl (*E*)-2-hexenoate: neat material (100 μl) and a lower dose (10 μl of neat material in 90 μl absolute ethanol). The trap transect included one trap baited with the lower concentration and two traps baited with the neat material, all positioned to alternate with three control traps having lures that contained 100 μl of ethanol. The experiment was replicated over 12 days. Lures were replaced every 3 days, and treatments were rotated one position along the transect every 4 days to control for positional effects. Traps were checked for beetles daily at ~1100 and 1800 hours.

The sex of adult *R. funebris* captured was determined by using the sexually dimorphic characters of antennal length and form of the fifth abdominal sternite (Linsley 1964). Differences between numbers of females and males captured were tested with the χ^2 goodness-of-fit test (Sokal and Rohlf 1995). Differences between treatments in numbers of beetles captured (sexes combined) were tested with the nonparametric Friedman's test (blocking by replicate; PROC FREQ with CMH option; SAS Institute 2001) because assumptions of analysis of variance were violated by heteroscedasticity (Sokal and Rohlf 1995). Differences between pairs of means were tested with the Ryan–Einot–Gabriel–Welsch (REGWQ) means-separation test to control maximum experiment-wise error rates (SAS Institute 2001). Six dates on which fewer than three beetles in total were captured were excluded from the analysis.

We also tested for the presence of adult *R. funebris* in a variety of different habitats in southern California (Table 1) by setting up pairs of traps: one baited with 100 μl of the 10% solution of synthetic (*Z*)-3-decenyl (*E*)-2-hexenoate in a plastic bag lure and the other a control trap with a solvent lure. These bioassays were conducted for varying periods between 28 June and 18 August 2008. Traps were checked for beetles every 1–5 days, and lures were replaced before the liquid had completely evaporated. Voucher specimens of *R. funebris* were deposited at the Entomology Research Museum at the University of California, Riverside.

Results

Identification of Pheromone Components Coupled GC–EAD analyses of headspace volatiles collected from live adult males of *R. funebris* showed that antennae of both sexes responded strongly to the major component in extracts from males (peak 4, Fig. 1). Several minor components in extracts elicited weaker responses from antennae of males (peaks 1–3, Fig. 1). The compounds (peaks 1–4) were produced in a ratio of 10.2±9.1:0.3± 0.3:15.3±9.9:100, respectively (mean ± standard deviation of four extracts), and all of these compounds were present only in extracts from males. Three aeration extracts from female beetles also were analyzed by GC–EAD, using antennae from three different males. The antennae did not respond to any components in the extracts, indicating that females of this species do not produce volatile sex pheromones, at least under the aeration conditions used in these studies.

GC–MS analyses of extracts showed that peak 1 (Fig. 1; Kovats index [KI] 1256 on DB-5 column) gave a weak molecular ion at *m/z* 156 (trace), with the first significant ion occurring at *m/z* 138 (12%) from loss of water, and a base peak at *m/z* 68 (for full mass spectrum, see Supplementary data). From comparison with database spectra and the retention times and mass spectrum of a (*Z*)-4-decenol standard (KI 1259), it appeared likely that this compound was a straight-chain decenol, with (*Z*)-3-decenol being the best match to database spectra. The spectrum of one of the two other minor components (peak 3, Fig. 1; KI 1770) and the major component (peak 4, Fig. 1; KI 1823) were very similar to each other, suggesting that

Table 1 Additional study sites in southern California where field bioassays were conducted (one trap baited with dilute [*Z*]-3-decenyl [*E*]-2-hexenoate and one control trap) in 2008 and the number and sex of adult *R. funebris* captured in baited traps

Location	Habitat	GPS coordinates (elevation)	Time period (# days)	♂/♀ captured
Kern Co., Mt. Pinos Rd.	Oak-conifer woodland	34°49′27″ N, 119°05′00″ W (1,969 m)	Late June–mid August (40)	2/1
Kern Co., Mt. Pinos Rd.	Oak-conifer woodland	34°49′18″ N, 119°05′27″ W (2,127 m)	Mid July–mid August (34)	0/1
Los Angeles Co., Big Pines Hwy.	Riparian area, oak-conifer woodland	34°22′57″ N, 117°42′00″ W (1,998 m)	Early July–mid August (27)	0/0
Los Angeles Co., State Hwy. 2	Oak woodland	34°22′46″ N, 117°43′44″ W (2,147 m)	Mid July–mid August (23)	0/0
Riverside Co., Residence	Urban forest	33°57′33″ N, 117°21′24″ W (290 m)	Early July (2)	1/1
Riverside Co., University of California Box Springs Reserve	Riparian area, coastal sage scrub, chamise chaparral	33°59′7″ N, 117°18′12″ W (416 m)	Early July (6)	1/1
Ventura Co., Mutau Flat Rd.	Oak-piñon pine woodland	34°42′37″ N, 119°07′15″ W (1,663 m)	Late July (5)	0/1

No *R. funebris* were captured in control traps at any site

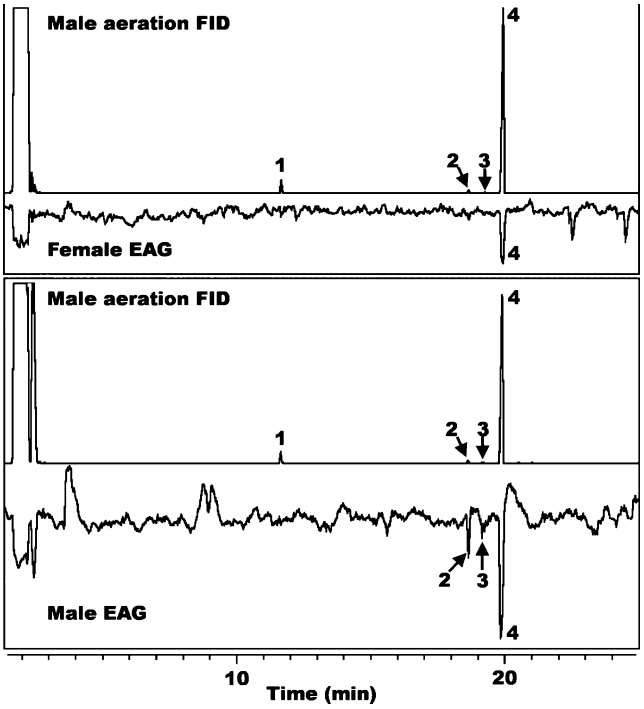

Fig. 1 Gas chromatogram (*top traces*) and corresponding electroantennogram (*EAG*) traces from antennae of female (*top*) and male (*bottom*) *Rosalia funebris*, in response to extract from a headspace collection of a male. A DB-5 column was used for coupled gas-chromatogram-EAD. A DB-5 column for the coupled gas chromatogram–EAG. *1* (*Z*)-3-decenol, *2* (*Z*)-3-nonenyl (*E*)-2-hexenoate, *3* (*Z*)-3-decenyl (*E*)-3-hexenoate, and *4* (*Z*)-3-decenyl (*E*)-2-hexenoate

they were isomers. The major, later-eluting isomer showed a weak molecular ion at *m/z* 252, with the first significant fragment at *m/z* 138 (74%), a strong peak at *m/z* 97 (95%), and a weaker fragment at *m/z* 115 (12%; for full mass spectrum, see Supplementary data). The latter two fragments would be expected from hexenoate esters so that, in total, these data suggested that the compound might be a decenyl hexenoate. Reduction of the crude extract with H_2 and a palladium-on-carbon catalyst gave a small, early-eluting peak (KI 1273) of which the mass spectrum and retention time matched those of decanol. The reduction collapsed peaks 3 and 4 into one peak (KI 1785) with the mass spectrum and retention time matching those of decyl hexanoate. These data provided support for the tentative identifications of peak 1 as a decenol and peaks 3 and 4 as decenyl hexenoate isomers. The fact that the retention time of the major component (peak 4) was significantly longer than that of decyl hexanoate on the nonpolar HP5-MS GC column suggested that the double bond in the hexenyl portion of the ester was conjugated with the carbonyl group.

The decenol component of the crude extract was confirmed as (*Z*)-3-decenol by epoxidation of crude extract. The retention times and mass spectra of both the underivatized (*Z*)-3-decenol and the epoxidized (*Z*)-3-decenol matched those of synthetic standards. Although we did not know which stereoisomer was correct a priori, these matches confirmed both the position and the geometry of the double bond (Hogge and Millar 1987). These data also suggested that the major component in the extract was a (*Z*)-3-decenyl 2-hexenoate, and the retention time and mass spectrum of a synthesized standard of (*Z*)-3-decenyl (*E*)-2-hexenoate matched those of the major component on both nonpolar DB-5 and polar DB-Wax columns. As with the determination of the double bond stereochemistry of (*Z*)-3-decenol described above, we did not know the stereochemistry of the conjugated acid portion of the ester a priori, but the retention time and mass spectral matches with the synthetic standard provided good evidence for (*E*)-2 stereochemistry. This stereochemistry was verified by epoxidation of the ester components in the insect extract. Although the epoxidation of the extract did not proceed cleanly, possibly due to acid-catalyzed rearrangements of the initial diepoxide products, epoxidation of pure synthetic (*Z*)-3-decenyl (*E*)-2-hexenoate resulted in a pattern of peaks that was qualitatively similar to those in the epoxidized insect extract, with matching retention times and mass spectra. Furthermore, antennae from beetles of both sexes responded strongly to synthetic (*Z*)-3-decenyl (*E*)-2-hexenoate in GC-EAD bioassays. Finally, the retention indices of (*Z*)-3-decenol and (*Z*)-3-decenyl (*E*)-2-hexenoate on a polar DB-Wax column (KI 1767 and 2168, respectively) exactly matched those of standards. In sum, these data confirmed that the major male-specific compound in the aeration extracts from male *R. funebris* was (*Z*)-3-decenyl (*E*)-2-hexenoate and that the early-eluting trace component (peak 1) was (*Z*)-3-decenol.

The ester isomer with the slightly lower KI value than the major component (KI value 1770; peak 3, Fig. 1) was tentatively identified as a deconjugated analog of the main compound (for full mass spectrum, see Supplementary data) because it was generated as an impurity during attempted esterification of (*E*)-2-hexenoic acid with (*Z*)-3-decenol using 4-(*N,N*)-dimethylaminopyridine and dicyclohexylcarbodiimide (Hasner and Alexanian 1978). This tentative identification was confirmed by synthesis of an authentic standard of (*Z*)-3-decenyl (*E*)-3-hexenoate, the retention time and mass spectrum of which matched those of the insect-produced compound. Fortuitously, the (*E*)-3-hexenoic acid starting material was contaminated with a small amount of the (*Z*)-isomer, the ester of which had a different retention time, so that the identification of the stereochemistry in the natural compound was unequivocal.

A partial mass spectrum was obtained for peak 2, and the fragments suggested that it was a homolog of the main component, with a nine-carbon alcohol component to the

ester. A molecular ion was not seen, but significant ions included *m/z* 124 (54), 115 (8), 97 (76), 82 (66), 68 (74), 55 (100), and 41 (53). The ions at *m/z* 97 and 115, corresponding to fragments from a hexenoate ester, indicated that it was the alcohol rather than the acid portion of the ester that was one carbon shorter than the equivalent portion in the major component, and the KI value of 1725, almost exactly 100 KI units less than that of the main component, provided further evidence that it was probably a homolog that was shorter by one carbon. The identification was confirmed by synthesis of an authentic standard (for full mass spectrum, see Supplementary data), with spectral and retention time data matching those of the insect-produced compound.

Field Bioassays of Synthetic Compounds The sentinel trap at Placerita Canyon Natural Area captured the first adult *R. funebris* on 22 June 2008. We subsequently captured 48 adults in the field bioassay (four males and 44 females; sex ratio significantly different from 1:1, $\chi^2=33.3$, $P<0.001$), with a maximum of 12 adults (including three of the four males) over a 24-h period in one trap baited with neat (*Z*)-3-decenyl (*E*)-2-hexenoate. The number of beetles trapped per day reached a peak of 18 after 1 week but fell to one and zero over the last 4 days. Treatment means were significantly different (Fig. 2; sexes combined, Friedman's $Q_{2,35}=18.2$, $P<0.001$). Traps baited with diluted and neat (*Z*)-3-decenyl (*E*)-2-hexenoate captured significantly more beetles than did controls (Fig. 2), but the pheromone treatment means were not significantly different from one another. All but two of the beetles were captured between 1100 and 1800 hour. Traps baited with synthetic pheromone did not capture any other insect species in consistent or significant numbers.

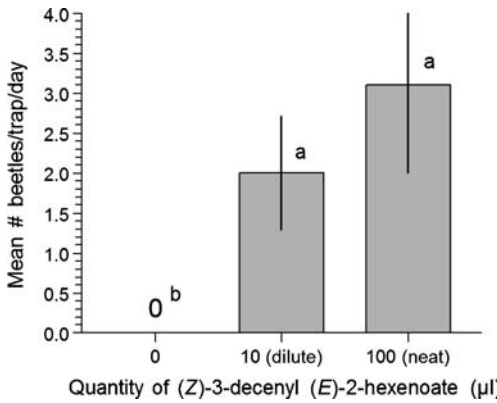

Fig. 2 Mean (± standard error) number of adult (sexes combined) *Rosalia funebris* captured from 22 June to 3 July 2008 at Placerita Canyon Natural Area in panel traps baited with 100 µl neat (*Z*)-3-decenyl (*E*)-2-hexenoate, 10 µl of (*Z*)-3-decenyl (*E*)-2-hexenoate in 90 µl of ethanol, or 100 µl of ethanol (solvent control). Bars with different letters are significantly different (REGWQ test $P<0.05$)

Traps baited with dilute (*Z*)-3-decenyl (*E*)-2-hexenoate captured a total of nine adult *R. funebris* (four males, five females) at additional study sites (Table 1), with no beetles being caught in control traps. Some of these sites yielded no beetles, even though baited traps were in place for several weeks (Table 1).

Discussion

Attraction of both sexes of *R. funebris* in field bioassays to lures baited with synthetic (*Z*)-3-decenyl (*E*)-2-hexenoate suggested that this compound is an aggregation rather than a sex pheromone, as appears to be true for pheromones produced by many other cerambycine species (Hanks et al. 2007; Lacey et al. 2008b). The strong female bias of adult *R. funebris* captured in the replicated field trial suggests that the sexes differ either in their sensitivity or their responses to the compound. However, it is impossible to draw conclusions about differential responses of the sexes to the pheromone based on these data without information about the sex ratio of the local population. Also, males that were captured alive in the traps may have emitted pheromone and so could have influenced subsequent trap catches of conspecifics. Nevertheless, most of the females were captured in traps that did not contain males, so their response to the pheromone treatment was unequivocal.

The small numbers of beetles captured at the additional sites suggest that our choice of study site for the field bioassays, Placerita Canyon Natural Area, was fortuitous and attest to the general rarity of *R. funebris*, as has been previously noted (Essig, 1943; Linsley 1964, 1995). It is possible that *R. funebris* was more abundant in that area because of recent wildfires. Studies on this species suggest that females oviposit in burned trees (IPS, personal observation). The brief period of time that adult *R. funebris* were captured in traps at Placerita Canyon Natural Area (~9 days) is consistent with other reports and observations that adults of this species are only active for a short period (Chemsak and Linsley 1971; IPS, personal observation).

That we captured 12 beetles in one trap in a single day indicates that (*Z*)-3-decenyl (*E*)-2-hexenoate is sufficient to attract *R. funebris* in significant numbers when adults are active. Nevertheless, it is possible that the minor components identified may form part of the natural pheromone and that traps baited with a more complete reconstruction of the blend could attract greater numbers of beetles. Unfortunately, the full identification and syntheses of the minor components was not completed in time for these chemicals to be included in the field trials.

The function of the aggregation pheromone of *R. funebris* is probably similar to that of other cerambycid species. Both sexes of many cerambycid species are attracted to volatile compounds emanating from patches of larval host plants (Hanks 1999; Ginzel and Hanks 2005). Once in a patch of host plants, volatile pheromones may further facilitate mate location by bringing males and females together on hosts (Ginzel and Hanks 2005). Nonvolatile contact chemical signals also play a role, once the sexes are in the same vicinity, in males identifying conspecific females (Ginzel and Hanks 2003, Ginzel et al. 2003a, b; Lacey et al. 2008a). Consistent with these behaviors for other cerambycid species, adult *R. funebris* of both sexes aggregate on larval hosts (Linsley 1995). Aggregation also may expedite host colonization, a distinct adaptive advantage, because the quality of larval hosts (dying trees) declines rapidly as hosts are colonized by other types of wood-boring insects and saprophytes (Hanks 1999).

The structure of the male-produced pheromone of *R. funebris* is unprecedented among the cerambycid pheromones that have been reported to date and unlike the diol/hydroxyketone motif of the volatile pheromones of most other cerambycine species (see "Introduction"). Although the diol/hydroxyketone pheromone motif is common, it is by no means universal among members of this large and diverse subfamily, as further indicated by the pheromones of *P. lecontei* and *M. caryae* (Hanks et al. 2007; Lacey et al. 2008b). The unique structure of the pheromone of *R. funebris* is consistent with this species being relatively distantly related to other cerambycines for which pheromones have been identified. *Rosalia funebris* is the sole representative of the tribe Rosaliini in the Western Hemisphere, whereas the other cerambycine species for which pheromones have been identified are in the tribes Anaglyptini, Callidiini, and Clytini (Ray et al. 2006; Hanks et al. 2007; taxonomy according to Monné and Hovore 2005). Furthermore, *R. funebris* lacks the distinctive prothoracic glands and gland pores that are the source of pheromones of other cerambycine species (Ray et al. 2006), and we do not yet know either the source or the mechanism of release of the *R. funebris* pheromone. The only other known pheromone glands of cerambycids that are not in the prothorax are those on the ovipositor of females of the more distantly related species *Prionus californicus* Mots. (Barbour et al. 2006; Cervantes et al. 2006).

To our knowledge, (*Z*)-3-decenyl (*E*)-2-hexenoate or any of the minor components of the volatiles produced by male *R. funebris* are NOT similar in structure to volatile compounds released by house paint. Therefore, it seems likely that the attraction of both sexes to newly painted structures, which has been observed and documented a number of times, may be due to some structural similarity among volatile compounds emanating from paint and compounds released by larval host plants, rather than similarity to the pheromone. It also seems unlikely that the observed attraction is due to color or other optical properties of the paint, which presumably persist over time, unlike the release of volatile chemicals from drying paint.

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Behavioral Evidence for a Contact Sex Pheromone Component of the Emerald Ash Borer, *Agrilus Planipennis* Fairmaire

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Abstract The cuticular hydrocarbon profiles of emerald ash borers, *Agrilus planipennis*, were examined to determine if there are differences in these compounds between the sexes. We also assessed feral male EAB in the field for behavioral changes based on the application of a female-specific compound to dead, solvent-washed beetles. Males in the field spent significantly more time attempting copulation with dead, pinned female beetles coated with a three-beetle-equivalent dose of 3-methyltricosane than with solvent-washed beetles or those coated in 3-methyltricosane at lower concentrations. Males in the field spent the most time investigating pinned dead, unwashed female beetles. In the laboratory, sexually mature males were presented with one of several mixtures applied in hexane to filter paper disks or to the elytra of dead female beetles first washed in solvent. Male EAB also spent more time investigating dead beetles treated with solution applications that contained 3-methyltricosane than dead beetles and filter paper disks treated with male body wash or a straight-chain hydrocarbon not found on the cuticle of EAB.

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Introduction

The emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) is a growing threat to the ash (*Fraxinus* sp.) resource of North America (reviewed in Poland and McCullough 2006). The beetle is spreading rapidly, and efforts to detect new infestations are of paramount importance to slowing further spread. Thus far, no species-specific trap is available for wide deployment. The use of girdled "trap trees" has proven to be an effective but cost- and labor-intensive method of detecting new infestations (Cappaert et al. 2005). Progress toward development of a widely deployable trap has been made on the grounds of trap color (Francese et al. 2005), with purple traps being the most effective at capturing adult EAB. Induced volatiles from ash trees are antennally active in adult EAB (Rodriguez-Saona et al. 2007) and incorporated into purple prism traps for a significant gain in trapping effectiveness (Crook et al. 2008). Green leafy volatiles derived from ash trees also have been identified and shown to improve the capture of adult EAB on traps (De Groot et al. 2008).

Field and the laboratory studies on the mating system of this beetle found that vision plays a key role in male mate-location (Lelito et al. 2007), similar to the mating systems of other buprestids examined to date (Carlson and Knight 1969; Matthews and Matthews 1978; Gwynne and Rentz 1983). Further, males appear to discriminate between the sexes once contact is made; feral males spend significantly

more time attempting copulation with females than males or solvent-washed beetles of both sexes, suggesting the use of a contact cue (Lelito et al. 2007).

In an effort to understand the role of any sex-specific compounds the beetle may employ in mate recognition, we conducted solvent dipping and SPME sampling of the cuticles of both immature and mature male, as well as female, beetles. These samples revealed characteristic differences between the sexes once they are mature. Here, we examine the behavioral role of 3-methyltricosane, a long-chain hydrocarbon present on the cuticles of mature females, but only in traces on the cuticles of male or immature female EAB. The compound was tested for behavioral activity in the field, using dead adult EAB as lures in a manner similar to that used in the past to identify precopulatory behavior (Lelito et al. 2007), as well as in a laboratory bioassay to assess the arrestant and/or attractant properties of the compound.

Methods and Materials

Insects Newly emerged adult beetles were provided by the staff of the Brighton, MI (USA 48116) USDA APHIS PPQ laboratory. These were segregated by sex upon emergence from rearing barrels, maintained in separate rearing tubs, and fed on ash foliage obtained from trees grown indoors. Beetles used for solvent and SPME sampling were either 3 ("young") or at least 10–12 days post-eclosion ("mature"). Live beetles used in laboratory behavior assessments were between 8 and 18 days of age and were considered "mature" (Bauer et al. 2004; Lyons et al. 2004). The beetles we used as field lures were also mature, killed by freezing, pinned through the thorax, and allowed to dry.

Solvent Dipping and SPME Sampling Young and mature beetles of both sexes were extracted in groups of three in 300 µl of dichloromethane (B&J, High Purity Solvent) or hexane (B&J, Ultra Resi-Analyzed). We did not detect significant differences between the dichloromethane and hexane washes of the beetles; therefore, we used the hexane solutions for quantification and further analysis. Hexane was evaporated in a gentle stream of nitrogen and samples redissolved in 60 µl of 50 ng/µl of 16-methyl hexatriacontane as an internal standard in hexane. For each of the four treatments, we performed four replicates.

The spatial distribution of cuticular compounds on the surface of mature males and females was investigated by using SPME fibers coated with 7 µm of polydimethylsiloxane (Supelco, Bellefonte, PA, USA). Body tagmata (head, thorax, and abdomen) of three mature EAB of each sex were sampled for 2 min each by rubbing a SPME fiber gently against the cuticle of the beetle. We prepared three replicate SPME samples of each body part. Beetles were held in place with soft forceps during fiber application.

Chemical Analysis All samples were analyzed with an Agilent 6890 GC-FID system equipped with an Equity-5 column (30 m×0.2 mm×0.2 µm; Supelco, Bellefonte, PA, USA) for quantification purposes. To identify components, selected samples were analyzed on an identical column in an Agilent 6890N GC coupled with a 5973N MSD system in EI mode (+70 eV). The oven temperature program was 50°C (1 min)−20°C/min−210°C−3°C/min−320°C (10 min) for all GC analyses. The temperature of the injector was held at 280°C, and the FID in the GC and the transfer line in the GC-MS were kept at 300°C. Samples were injected splitless (0.75 min) and run at an average linear flow velocity of 25 cm/s in the GC and 30 cm/s in the GC-MS. SPME samples were analyzed using the same settings.

Identification of the compounds was based on their MS spectra (NIST05, Masslib), their Kovats indices on the Equity-5 column described elsewhere (Böröczky et al. 2008), and by comparison with authentic standards. Quantification of hydrocarbons was based on their peak area values obtained from our data acquisition and analysis software (Chemstation, Agilent). Peak area values were corrected with the relative response factors described elsewhere (Böröczky et al. 2008). Percent composition was calculated as percentage of the sum of all identified compounds. Absolute amounts were calculated relative to the internal standard. The FID response was linear in the concentration range of the compounds we analyzed.

Synthesis of 3-Methyltricosane A solution containing 0.78 g (2.8 mmol) of eicosanal in 30 ml of anhydrous ether was added to an excess of ethereal 2-butyl magnesium bromide under an argon atmosphere. The mixture was stirred overnight and after careful addition of 10% HCl; the ether layer was separated, washed with saturated $NaHCO_3$, and dried over anhydrous $MgSO_4$. After filtration, the residue was taken up in 7 ml of pyridine and treated with 0.31 ml of methane sulfonyl chloride at 0°C and stirred overnight. After the addition of 50 ml of ether, the mixture was washed with 10% HCl; the ether layer was separated, washed with saturated $NaHCO_3$, and dried over anhydrous $MgSO_4$. After filtration, the solvent was removed in vacuo, and the residue was taken up in 50 ml of ethyl acetate and hydrogenated over 100 mg of PtO_2 under 3 Atm of hydrogen overnight. After the mixture was filtered and the solvent was removed, flash chromatography (silica gel/hexane) provided 521 mg of 3-methyltricosane, *m/z* 338 [M^+](0.2), 309(10), 281(2), 25312), 239(2), 225(3), 211(3), 197(3), 183(3), 169(4), 155(5), 141(7), 127(9), 113(13), 99 (19), 97(9), 85(41), 83(9), 71(65), 69(14), 57(100), 56(36), 55(22), 43(57), 41(24). GC-MS was carried out in the EI

mode using a Shimadzu QP-2010 GC-MS equipped with an RTX-5, 30 m×0.25 mm i.d. column. The instrument was programmed from 60°C to 250°C at 10°C/min and held at 250°C for 40 min.

The synthetic compound had a Kovats index (2371 on the Equity-5 column used above) and MS spectrum identical to that of the compound found in the body washes.

Field Behavior The dead, pinned female "beetle-lures" used for these experiments were either: unwashed, "U"; washed in dichloromethane for 10 min and then dried for 24 h, "W"; or solvent-washed, dried, and then coated with one of our experimental treatments. Ten microliters of 4 ng/μl *n*-eicosane ("E"; an impurity resulting from our synthesis of 3-methyltricosane); 2 ng/μl 80%/20% 3-methyltricosane/*n*-eicosane mixture ("T1"); 6 ng/μl 80%/20% 3-methyltricosane/n-eicosane mixture ("T2"); or 20 ng/μl 80%/20% 3-methyltricosane/*n*-eicosane mixture ("T3") were applied to the dorsal cuticle of a dead, dichloromethane-washed, and dried female EAB. T1, T2, and T3 represented 16, 48, and 160 ng of 3-methyltricosane, respectively. All applications were made with a 1-μl glass pipette, which was cleaned between applications by three separate washes with 10 μl of hexane. We prepared fresh beetle-lures for each replicate of the field experiment.

Field experiments took place between 7 June and 5 July 2007 between the hours of 1000 AM and 1600 PM EST, on days without heavy rain. Each 2-h replicate (one per suitable day, for a total of 17 replicates) was performed in an area of high-density EAB population located in Livingston County, MI, USA, south of the town of Howell. The site used was private agricultural and forest land, containing a large number of green ash trees (*F. pennsylvanica*). Experiments were conducted prior to and through the peak EAB flight period. Individual ash trees selected for these experiments were between 10 and 20 m tall and had healthy branches accessible at 2–3 m height from ground level for the pinning of beetle-lures. Each tree used was selected randomly on each day, with the precondition that at least five live EAB could be seen on that tree from ground level (i.e., a presumably infested tree).

For each replicate of field experiment, we pinned three beetle-lures of each treatment (U, W, E, T1, T2, and T3), on individual terminal leaflets of compound ash leaves on the sunny side of a selected ash tree, between 2 and 3 m from ground level. Beetle-lures were positioned randomly (not blocked by treatment), between 10 and 30 cm apart. We then observed these lures from the ground for a 2-h period and timed the duration of any feral EAB attempts at copulation and investigation of a lure. We defined "investigation" as a feral EAB remaining in contact with a pinned beetle-lure subsequent to an airborne approach and copulation attempt.

Laboratory Behavior We prepared experimental arenas by using a plastic 100-mm diameter Petri dish (Cat. No. 08-757-12, Fisher Scientific) lined with a 100-mm diameter filter paper insert (Cat. No. 1001-100, Whatman). For each experiment, we applied 10 μl of hexane as a control to the filter paper at each of two opposite points along the diameter of the dish, both 40 mm from the center of the filter paper. We then applied 10 μl of one of the experimental solutions (in hexane; Table 1) to the paper at the other two opposite points along the perpendicular diameter. We prepared both

Table 1 The cuticular lipid treatments prepared for the laboratory arena bioassays on *Agrilus planipennis,* utilizing a Petri dish arena and applications of listed solutions to filter papers and dead beetles

Beetle equivalents	Lure treatment	Dosage	Abbreviation
3[a]	*n*-eicosane (99%, Aldrich)	4 ng/μl *n*-eicosane	EIC3[b]
3	Female EAB Body Wash (in hexane)	12 Female EAB/100 μl hexane	FBW3
3	Male EAB Body Wash (in hexane)	12 Male EAB/100 μl hexane	MBW3
3	*n*-tetracosane (99%, Aldrich)	16 ng/μl *n*-tetracosane	TET3
3	80% 3-methyltricosane/20% *n*-eicosane	20 ng/μl 3-methyltricosane	TRM3[c]
3	91% 3-methyltricosane/9% *n*-eicosane	20 ng/μl 3-methyltricosane	TRP3
1[a]	*n*-eicosane (99%, Aldrich)	1.2 ng/μl *n*-eicosane	EIC1[b]
1	Female EAB Body Wash (in hexane)	4 Female EAB/100 μl hexane	FBW1
1	Male EAB Body Wash (in hexane)	4 Male EAB/100 μl hexane	MBW1
1	*n*-tetracosane (99%, Aldrich)	4.8 ng/μl *n*-tetracosane	TET1
1	80% 3-methyltricosane/20% *n*-eicosane	6 ng/μl 3-methyltricosane	TRM1[d]
1	91% 3-methyltricosane/9% *n*-eicosane	6 ng/μl 3-methyltricosane	TRP1

[a] Solution (10 μl) are applied to filter paper disk or solvent-washed dead EAB; beetle-equivalent rate determined from solvent-wash analysis of adult *A. planipennis* cuticular lipids

[b] Mixture identical to treatment 'E' from the field behavior experiment; dosage of "EIC3" matches "E"

[c] Mixture identical to treatments "T1," "T2," and "T3" from the field behavior experiment; matches dose of "T3"

[d] Mixture identical to treatments "T1," "T2," and "T3" from the field behavior experiment; matches dose of "T2"

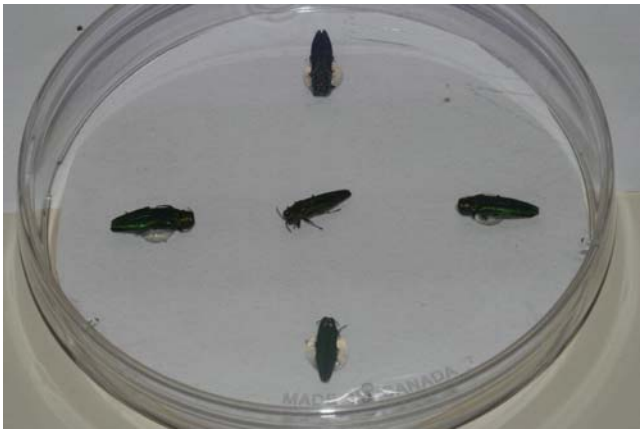

Fig. 1 The Petri dish used as an experimental arena for the *Agrilus planipennis* laboratory behavioral assay; the *beetle in the center* is a live male at the release point. The *other four beetles* are the dead, solvent-washed female beetles used as dummies for the application of either solvent controls or experimental lipid applications

male and female body washes by placing 12 mature individual EAB into 100 μl of hexane in a 2-ml glass vial, gently agitating for 2 min, and extracted the remaining liquid. We used the same approach for the lower dosage of one beetle-equivalent (1BE) but only extracted four mature beetles in 100 μl of hexane. We prepared fresh body wash solution at both dosages as needed and stored any remaining solution in a capped 2-ml glass vial in a standard freezer.

We performed 25 trials of each treatment at three and one BE. A new arena was prepared at the start of each trial, and the assay began when we placed individual mature (8–18 days post-eclosion) male EAB into the dish at the center-point of the filter paper. Each EAB male was then observed for a period of 10 min, while we recorded the number and duration of entries into the 1-cm diameter area centered on the application points.

We repeated the same assay but this time the solutions were applied to the dorsal cuticle of a dead, solvent-washed female EAB that had been hot-glued to the filter paper at the typical application points (Fig. 1) less than 24 h before and stored in a freezer prior to use. We recorded the number of direct male to female-beetle-lure contacts, the duration of each contact, and the number of times the male attempted to copulate.

We also conducted a series of trials to test mature female EAB for their response to beetle-lures using the same experimental treatments for mature males at the 3BE dosage described above. We recorded the number and duration of female-to beetle-lure contacts for each treatment.

A total of 25 individual 10-min trials were conducted for each treatment/lure combination (e.g., 3BE dosage, filter paper application) from 10 February to 15 March 2008, between 900 AM and 1400 PM EST. Three trials were run concurrently, with arenas approximately 10 cm apart under full-spectrum fluorescent lighting at 25°C. The lighting was situated on wooden supports such that all arenas were lit from directly overhead the center of the arena from a height of approximately 40 cm. Within each experiment, treatments were completely randomized within a day so that all treatments had an equal chance of occurring at a given time of day. The live EAB of both sexes were used only once, and following the assay were frozen and discarded.

For the laboratory experiments and the field experiments outlined above we purchased *n*-eicosane and *n*-tetracosane from Aldrich Chemical Co. (99% purity, St. Louis, MO, USA) and synthesized the 3-methyltricosane mixtures as outlined above.

Statistical Analyses We compared the mean investigation time of each treatment of beetle-lure in the field experiment time using a two-way ANOVA (analysis of variance), with replicate and treatment of the lure as factors. Treatment means were separated by in pair-wise comparisons by Tukey's Honestly Significant Differences test. We employed a binomial test of proportions to detect any treatments that were contacted more often than their paired hexane control (a significant deviation from a 50–50 ratio could indicate either attraction to, or avoidance of, a given treatment). Laboratory bioassay data were log-transformed prior to analysis to achieve a normal distribution. We utilized PROC GLM in SAS for comparisons of mean times of investigation between treatments by male EAB, using both the lure type and the chemical treatment of the

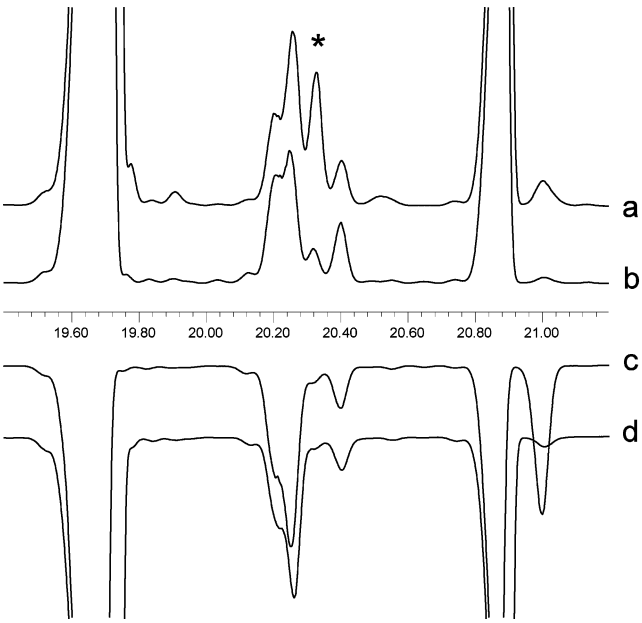

Fig. 2 The section of the gas chromatographic profile of the hexane body wash of mature *Agrilus planipennis* females, *a* young females, *b* young males, *c* and mature males, *d* containing 3-methyltricosane (indicated by the *asterisk*). 3-Methyltricosane is a minor component of the cuticular lipid profile of mature females; it occurs as a trace, if at all, in male and immature female beetles

Table 2 Quantification of selected cuticular hydrocarbons in the hexane body wash of young and mature adult *Agrilus planipennis* of both sexes

	Amount (ng/beetle)							
	Males				Females			
	3-day-old		12-day-old		3-day-old		12-day-old	
	Mean[a]	S.E.M.	Mean[a]	S.E.M.	Mean[a]	S.E.M.	Mean[a]	S.E.M.
5-Methyltricosane	tr		tr				6	1
3-Methyltricosane	4	1	5	1	8	1	47	10
5-Methylpentacosane	10	1	14	2	13	3	42	14
3-Methylpentacosane	15	1	23	3	22	3	68	14
5-Methylheptacosane	14	1	22	3	17	3	35	11
3-Methylheptacosane	30	3	38	5	39	7	86	20
3-Methyluntriacontane	tr		29	3	tr		26	3
3-Methyltritriacontane	tr		26	3	tr		22	2
Total alkanes	28,647	2,572	4,5717	6,110	3,2237	4,275	33,487	5,503

tr Amount is 1 ng/beetle or lower
[a] Four replicates, three beetles per replicate

lure as factors with investigation time as the dependent variable. We performed all statistical analyses in SAS Version 9.1.3 (SAS Institute 2006).

Results

Solvent Dipping and SPME Sampling Major components of the cuticle of both male and female EAB were found to be saturated and monomethyl branched odd-chain (C23–C29) hydrocarbons. The methyl branch typically was in the middle of the chain. Terminally branched monomethyl alkanes, dimethyl alkanes, and unsaturated hydrocarbons were minor components. More polar lipids, such as fatty acids, ethers, and acetate and butyrate esters of long-chain alcohols, were also detected in the body wash samples of both sexes.

There were characteristic differences between the cuticular chemistry of mature male and female beetles (Fig. 2). 3-Methyltricosane is a minor component of the female body wash but is present in only trace amounts in that of males. Furthermore, it was found in limited quantity on the cuticle of the young female EAB, but it increased to approximately 50 ng of material coincident with sexual maturity. Other terminally branched mono-methylalkanes showed a similar trend but not as strongly as 3-methyltricosane (Table 2). We did not detect any variation in expression of 3-methyltricosane between the tagmata of the females with SPME.

Field Behavior Replicates were not a significant source of variation (ANOVA, df=16, F=1.65, P=0.104), and male EAB flew to and landed on all types of lures equally (ANOVA, df=5, F=0.536, P=0.739). However, there was a significant treatment effect (ANOVA, df=5, F=20.66, P<0.001) once contact was made, with unwashed females being investigated significantly longer than all other lures (Fig. 3). Treatments T3 and E were investigated for significantly longer than the washed female control, while T1 and T2 were not (Fig. 3).

Laboratory Behavior Both lure type (ANOVA, df=1, F=12.30, P<0.001) and treatment (ANOVA, df=25, F=4.34, P<0.001) had a significant effect on male behavior so data

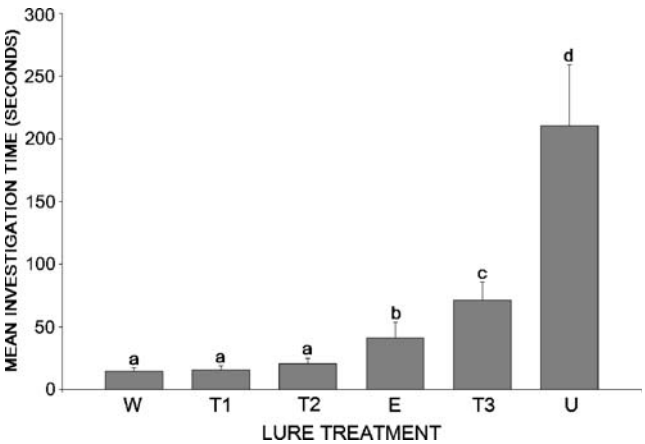

Fig. 3 Columns represent the mean time (in seconds) spent by feral male *Agrilus planipennis* in investigation of beetle-lures in the field in response to different treatments during each 2-h period of observation, with a total of 17 replicates. Treatments are listed in the following order on the x-axis: *W* a solvent-washed dead female EAB; *T1* 2 ng/μl 3-methyltricosane mixture application onto a washed female EAB; *T2* 6 ng/μl 3-methyltricosane mixture application; *E* 4 ng/μl eicosane application; *T3* 20 ng/μl 3-methyltricosane mixture application; *U* a nonsolvent-washed dead female EAB. *Bars* represent one standard error of the mean. *Columns having no letters in common* are significantly different (df=5, F=19.73, P<0.001). This experiment was performed between 7 June and 5 July 2007 South of Howell, Michigan

Table 3 Mean investigation time (seconds ± S.E.M.) by treatment in the male *Agrilus planipennis* laboratory arena bioassay

Treatment	Beetle-lure application[a]	Filter-paper droplet application[a]
EIC3	23.8±4.3ab	24.5±4.7ab
FBW3	53.4±10.6b	39.8±5.8b
MBW3	22.6±1.8ab	16.6±2.2a
TET3	35.3±7.2ab	27.5±4.7ab
TRM3	49.0±8.2b	32.7±6.8ab
TRP3	45.1±3.1b	23.6±2.9ab
Hexane[b]	22.1±1.0a	19.0±0.8a
EIC1	24.1±5.5a	19.5±2.2ab
FBW1	39.6±7.0ab	22.6±3.7ab
MBW1	23.7±4.1a	20.0±1.7ab
TET1	24.3±2.9ab	21.8±3.2ab
TRM1	29.5±2.9ab	25.9±4.5ab
TRP1	25.5±2.8ab	22.1±3.9ab
Hexane[b]	22.1±1.0a	19.0±0.8a

The bioassay utilized droplet applications to filter paper discs and dead, solvent-washed *A. planipennis* adults

[a] Pooled control value. Individual controls were analyzed pairwise with Tukey's HSD against the experimental treatments. Individual controls paired with each experimental treatment did not vary statistically from one another by ANOVA, $P>0.05$

[b] Means within a column having no letters in common are significantly different according to Tukey's HSD, $P<0.05$

were separated by lure type and analyzed separately for treatment effects by using a one-way ANOVA. The chemical treatment applied to the lure had an effect on male behavior for both beetle-lures (Table 3; ANOVA, $df=12$, $F=4.79$, $P<0.001$) and filter paper applications (Table 3; ANOVA, $df=12$, $F=2.72$, $P=0.002$). A greater number of cuticular lipid applications onto dead female EAB (treatments FBW3, TRM3, TRP3; Table 3) evoked significant differences in male behavior over their respective controls than did the corresponding lipid applications directly onto filter paper (treatment FBW3 only; Table 3).

The number of contacts made by male EAB with the different beetle-lures or the number of male EAB entering the treated areas on the filter paper, did not differ significantly between treatments (binomial test of proportions, $P>0.05$ for all treatment-control pairs). However, treatment affected the incidence of copulation attempts to beetle-lures (ANOVA, $df=12$, $F=6.032$, $P<0.001$) with FBW3 and TRP3 eliciting more than other treatments and hexane-only controls (Fig. 4, black columns). At 1BE, no significant differences were observed (Fig. 4, grey columns).

Female EAB did not exhibit a significant preference for any treatment either with respect to initial contact (Binomial test of proportions, $P>0.05$ for all treatment-control pairs) or time remaining at the lure (ANOVA, $df=6$, $F=0.472$, $P=0.829$).

Discussion

Although the approach phase of the EAB mating system relies on visual cues, our results confirm the earlier suggestion that a female contact sex pheromone influences male behavior (Lelito et al. 2007). Males in the laboratory spent more time investigating beetle-lures to which we had reapplied female body wash or synthetic mixtures containing 3-methyltricosane than solvent-only controls, a preference we observed among feral male EAB tested in the field as well. However, males still spent significantly more time investigating unwashed female EAB than any dosage of 3-methyltricosane. Therefore, it is likely that the behavioral effect of 3-methyltricosane may be synergized by the perception of other compounds naturally secreted onto the cuticular surface of the unwashed beetle-lures.

The results of our assays support the pheromonal role of the cuticular hydrocarbon 3-methyltricosane, and the identification of this compound represents the first contact

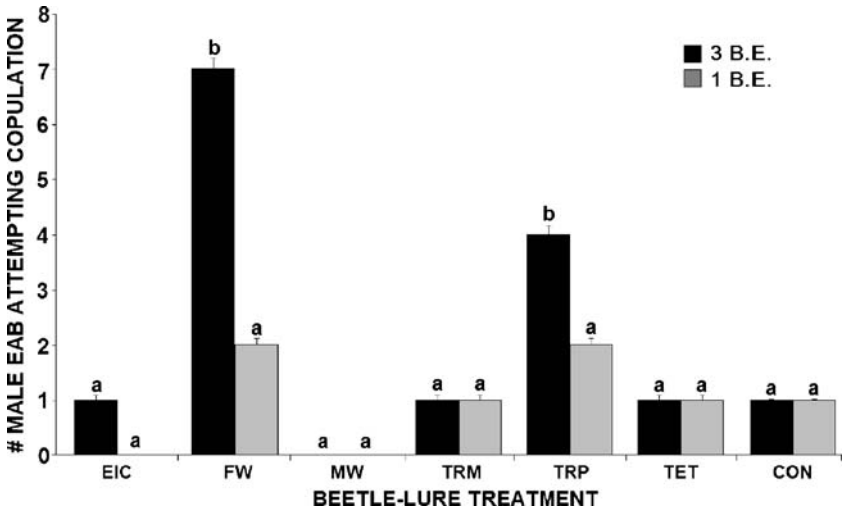

Fig. 4 Columns represent the total number of copulation attempts performed by male *Agrilus planipennis* to each experimental treatment of beetle-lure. *Black columns* indicate three beetle-equivalent dosage, *grey columns* indicate one beetle-equivalent dosage. Treatment abbreviations on the x-axis are the same as in Fig. 4. *Bars* represent two standard errors of the mean. *Columns having no letters in common* are significantly different from one another ($df=12$, $F=6.03$, $P<0.001$)

sex pheromone identified in the family Buprestidae. However, this compound has a chiral center at position 3, and it will be necessary to synthesize and test both enantiomers to determine if chirality is important.

Males do not differentiate over a distance between beetles treated with 3-methyltricosane and those treated with female body wash or solvent only in either the laboratory or the field, suggesting that 3-methyltricosane is likely perceived only as a contact cue. Although it is possible that males might be able to detect the presence of females from outside their visual range either directly or indirectly, through olfactory cues such as volatiles given off by the damaged host (Crook et al. 2008), 3-methyltricosane is unlikely to serve as a long-range attractant.

The continuous antennation of the treated substrate, especially obvious when the male encountered an affixed beetle to which female extract or 3-methyltricosane had been applied, suggests that the specific sensillae are on the antennae. Male chemoreception may not be limited to the antennae: males were also observed to "scratch" their tarsi against both the filter paper and the affixed beetles in many cases. This behavior was often followed by renewed bouts of vigorous antennation. This needs to be examined in greater detail.

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Development of an Efficient Pheromone-Based Trapping Method for the Banana Root Borer *Cosmopolites sordidus*

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Abstract The banana root borer *Cosmopolites sordidus* (Germar) (Coleoptera: Curculionidae) is a major pest of bananas throughout the world. Chemical control is both undesirable and expensive, where biological control alternatives are limited, and pheromone-based trapping results in low captures. In this study, several important factors that affect pheromone-based catches, such as trap type, trap dimensions, and color and position of the traps, were optimized. Ground traps were found to be superior to ramp and pitfall traps, and larger traps (40×25 cm and above) were more efficient than smaller ones (30×15 cm). In a color-choice test, the banana weevil clearly preferred brown traps over yellow, red, gray, blue, black, white, and green, with mahogany being more attractive than other shades of brown. In addition, pheromone baited ground traps positioned in the shade of the canopy caught significantly more adults than those placed in sunlight. Therefore, mahogany-brown ground traps 40×25 cm appear to be the most efficient at catching *C. sordidus* adults and have the greatest potential for use in mass trapping and programs for eradication of this pest.

Keywords Aggregation pheromone · Traps · Color preference · Banana root borer · *Cosmopolites sordidus* · Coleoptera · Curculionidae

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Introduction

The banana root borer *Cosmopolites sordidus* (Germar) (Coleoptera: Curculionidae) is native to Malaysia and Indonesia but is now a major pest in most banana-growing regions of the world (Gold et al. 2001), particularly those producing dessert and cooking bananas (Sikora et al. 1989). In recent times, this borer has become a serious problem in Guam and other parts of Micronesia, raising concern among local authorities because all varieties of bananas appear to be attacked, and losses may reach 100% if the pest is left uncontrolled (Koppenhöffer et al. 1994).

Budenberg et al. (1993) provided the first evidence of a male-produced aggregation pheromone in *C. sordidus*, and Beauhaire et al. (1995) detected six male-specific compounds that elicited electroantennogram activity in vapor form, including the male pheromone (sordidin; 1S,3R,5R, 7S-1-ethyl-3,5,7-trimethyl-2,8-dioxabicyclo[3.2.1]octane). The structure and absolute stereochemistry of the natural attractant were confirmed by synthesis (Mori et al. 1996; Fletcher et al. 1997) and racemic sordidin proved attractive to both sexes (Ndiege et al. 1996; Jayaraman et al. 1997). Sordidin, formulated by ChemTica Internacional, S.A., San José, Costa Rica, is used to monitor the banana borer (Tinzaara et al., 2002, 2003) in Costa Rica (Alpizar et al. 1999; Oehlschlager et al. 2000) and Uganda (Tinzaara et al. 2000). In the Caribbean, four baited pitfall traps/hectare reduced corm damage (to <10%) and increased bunch weights (by 10–20% over a crop cycle) in commercial plantations (Alpizar et al. 1999, 2000). However, in Africa, the use of sordidin was ineffective for mass trapping (Tinzaara et al. 2005). Therefore, there is an urgent need for an efficient semiochemical-based trapping method for the control of *C. sordidus*. Here, we report a study directed toward improving trapping methods to control the pest and also toward a better understanding of the pheromone ecology of the pest.

Methods and Materials

Trap Types Three different types of traps—ground, ramp, and pitfall—were evaluated. The ground trap (Fig. 1A) was constructed in our laboratory from a 120×60×0.5-cm piece of white corrugated plastic board, with a 50×8-cm slit baffle fitted at the top to prevent borers from escaping (Reddy et al. 2005). Traps were sealed in all four corners and along edges with marine adhesive sealant, and water, mixed with a dishwashing liquid detergent (1–3%), was put in the bottom container to retain adults. The lower outer edges of the ground traps were covered with earth to prevent weevils from crawling under the traps.

The ramp trap used was commercially available from ChemTica Internacional S.A. (San José, Costa Rica; Fig. 1B). It was made of durable polyethylene and consisted of two box-shaped components, each 14 cm wide by 4 cm high (inside dimensions). Four sloping ramps led from the four cardinal directions into the sides of one of the open boxes. Each ramp was 4 cm high, 13 cm long, and 12 cm wide and slid into a slot in the floor of the box component. The other box had four corner ridges (6 cm high) that extended downward into the bottom box. As in the ground trap, the bottom container was filled partially with soapy water to retain attracted borers.

Pitfall traps were cylindrical, translucent white plastic cups (10-cm diameter, 1.5-L capacity; Fig. 1C). Four drainage holes (24-mm diameter) were drilled at 90° from each other in the sides of the cup, at least 5 cm from the bottom. The pheromone lure was suspended from the top of the cup on a vinyl-clad steel wire (12 cm long) threaded through a 3-mm hole. Traps were placed in holes dug 10 cm into the ground so that the upper edge of the cup was at the level of the soil.

Pheromone Lures Pheromone lures (Cosmolure), sealed in a polymer membrane release device and optimized for *C. sordidus*, were obtained from ChemTica Internacional S.A. (San José, Costa Rica). The lure packs, each containing 90 mg of pheromone and having a release rate of 3 mg/day (Tinzaara et al. 2005), were stored at 4°C until use. Lures were hung on 2-cm wires suspended across the tops of the ground and pitfall traps. In the case of the ramp trap, a hook-shaped wire with the lure was inserted (2 cm) through a hole in the top side of the trap. The lures were changed when the transparent container with the pheromone appeared empty, usually once or twice a month, although occasionally more frequent changes were necessary.

Effect of Trap Type on Capture Efficiency Ground, ramp, and pitfall traps with synthetic pheromone lures were

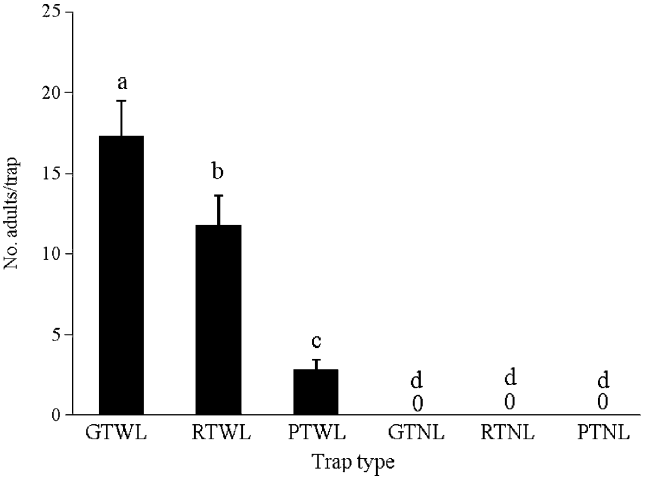

Fig. 2 Mean (± SE) numbers of adult *Cosmopolites sordidus* caught in ground, ramp, and pitfall traps baited with pheromone lures and in unbaited controls. *Different lower-case letters* indicate significant differences between treatments (one-way ANOVA using Poisson model, Least Square Means, $P<0.001$). *GTWL* ground trap with lure; *RTWL* ramp trap with lure; *PTWL* pitfall trap with lure; *GTNL* ground trap without lure; *RTNL* ramp trap without lure; *PTNL* pitfall trap without lure

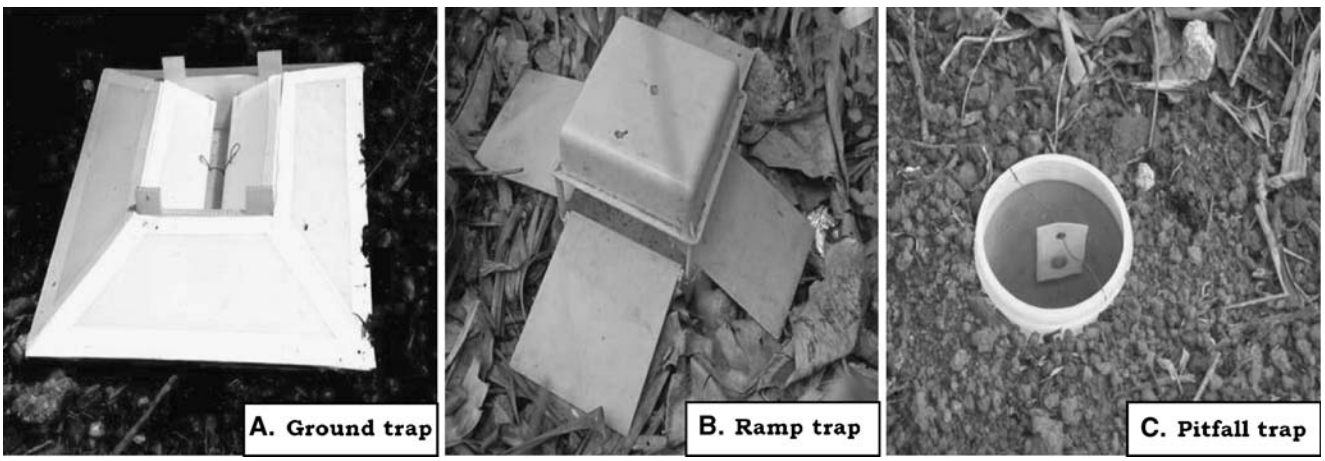

Fig. 1 **A–C** Design of the three traps used in experiments on *Cosmopolites sordidus*

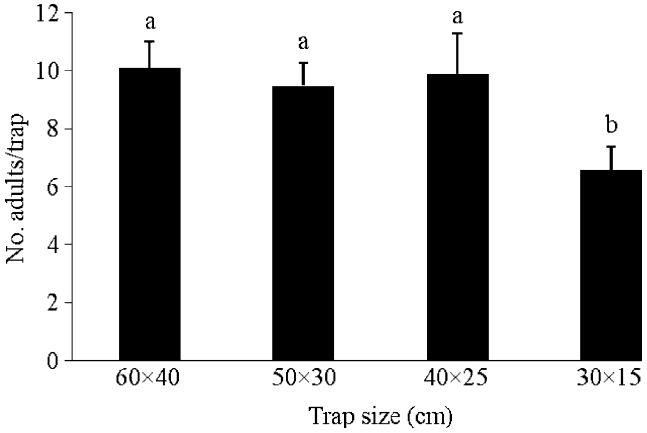

Fig. 3 Mean (± SE) numbers of adult *Cosmopolites sordidus* caught in pheromone-baited ground traps of different sizes. *Different lowercase letters* indicate significant differences between treatments (one-way ANOVA using Poisson model, Least Square Means, $P<0.05$)

placed at randomly chosen locations about 5 m apart on the ground of banana plantations in the villages of Dededo, Yigo, Agaña, and Yoña (Guam, USA; replicated ×4 at each site). Every week, the banana root borers trapped were removed and counted. Traps were washed and rinsed, and new detergent water was added. We randomized the traps across the field to avoid any possible trap-placement effect. Traps without lure were used as controls. The experiment was carried out from January to March 2007.

Effect of Trap Size The relative efficacy of four different sized ground traps (60×40, 50×30, 40×25, and 30×15 cm) was investigated. The ground trap was selected because it caught significantly more weevils than the ramp and pitfall traps. In each village, one trap of each size was set up, and their position rotated every week to prevent any location effect. The experiment was conducted from April to June 2007.

Effect of Trap Color Brown-, black-, gray-, yellow-, red-, white-, green-, and blue-colored vinyl tapes were adhered to all sides of 40×25-cm ground traps and tested independently (one trap of each color per location). The experiment was carried out from July to August 2007. The hue angle (h°) and chroma (C*) and the average of three readings for each color were determined by using a Konica Minolta CR-410 Chromameter (Minolta Instrument Systems, Ramsey, NJ, USA). The hue angle indicates the sample color, whereas the chroma provides a measure of the color intensity, and these were calculated by using the equations of Wrolstad et al. (2005). The hue angle is expressed on a 360° grid where 0°=red, 90°=yellow, 180°=green, and 270°=blue, respectively.

Effect of Shade of Brown Brown traps caught significantly more adults than traps of other colors so ground traps of different shades of brown (dark brown, mahogany brown, russet brown, saddle brown, and light brown) were evaluated (one trap of each shade in each village). The experiment was conducted from September to November 2007.

Relative Effects of Visual and Olfactory Cues To determine the relative importance of the visual and olfactory components of attraction, we repeated the experiment with mahogany brown ground traps baited with pheromone lures or unbaited. There were four replicates in each village, tested from December 2007 to February 2008.

Effect of Trap Shading Baited mahogany brown ground traps were placed in the shade and in sunlight, with four replicates in each village from March to May 2008. During the experiments, the color of the trap was measured by using a chromameter in the morning (0830 hours), noon (1200 hours), and afternoon (1600 hours). The data were averaged, and means were compared. Similarly, the

Table 1 Color measurements of traps used in the trapping of *C. sordidus* in Guam

Trap color	L*	a*	b*	Chroma (C)	Hue angle (h°)
Black	30.44±0.06	0.42±0.03	−1.08±0.04	1.16±0.05	–
Brown	35.26±0.18	3.98±0.03	3.94±0.02	5.60±0.03	44.66±0.11
Gray	39.83±0.11	−0.17±0.02	−2.23±0.01	2.24±0.01	85.64±0.47
Yellow	82.57±0.02	−2.92±0.03	84.02±0.27	84.07±0.27	91.99±0.02
Red	42.84±0.11	49.88±0.28	19.44±0.20	53.54±0.34	21.29±0.09
White	92.29±0.03	1.34±0.01	−2.59±0.04	2.91±0.03	–
Green	43.50±0.08	−27.32±0.03	1.72±0.09	27.37±0.03	176.39±0.19
Blue	36.02±0.10	15.19±0.10	−35.82±0.12	38.91±0.14	292.98±0.08

Data represent means of three observations

*L** indicates lightness of the color and it runs through the center of the color chart, where 100 at the top represents white and zero at the bottom represents black, *a** runs left to right on the color chart, and indicates a red shade when greater than zero (positive) and a green shade when lower than zero (negative), *b** runs vertically through the color chart, and indicates a yellow shade when positive and a blue shade when negative

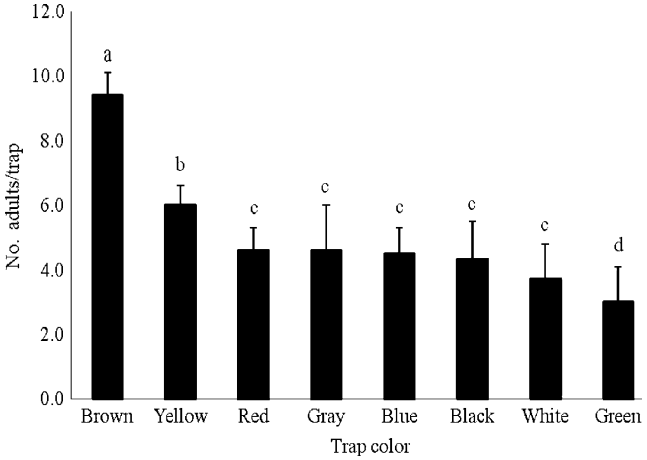

Fig. 4 Mean (± SE) numbers of adult *Cosmopolites sordidus* caught in pheromone-baited ground traps of different colors. *Different lowercase letters* indicate significant differences between treatments (one-way ANOVA using Poisson model, Least Square Means, $P<0.001$)

temperature within the different traps was recorded using a pocket weather meter (Kestrel®3000, Boothwyn, PA, USA).

Statistical Analysis Because all the response variables used in the experiments are count variables, a one-way Poisson analysis of variance model was fitted using the GLIMMIX Procedure SAS Version 9.13 (SAS Institute Inc. 2004). The Least Square Means test was used to make multiple comparisons for significant differences between treatments.

Results

Effect of Trap Type Baited ground traps captured more adult borers than baited ramp or pitfall traps ($F=48.88$, $df=2$, $P<0.001$; Fig. 2). Control traps captured no adult borers.

Effect of Trap Size The three larger trap sizes performed equally ($F=3.59$, $df=3$, $P<0.05$) but were significantly better than the smallest one (mean of 10.1±0.9, 9.5±0.8 and 9.9±1.4 adults/trap for 60×14, 50×30, and 40×25 cm, respectively; Fig. 3). For economy and ease of handling, 40×25-cm traps were chosen for further study.

Effect of Trap Color Trap color measurement values (L*, a*, b*, chroma, and hue angle) are given in Table 1. Brown ground traps were more attractive to banana borers than those of any other color tested ($F=6.74$, $df=7$, $P<0.001$; Fig. 4). There was a significant difference ($P<0.001$) between yellow and red colors. For the other colors, yellow attracted more banana borers than red, gray, blue, black, white, and green.

Effect of Shade of Brown Trap color measurement values (L*, a*, b*, chroma, and hue angle) for the different shades of brown are given in Table 2. The shade of brown affected adult catches in ground traps ($F=7.31$, $df=4$, $P<0.001$; Fig. 5), with mahogany brown traps catching significantly more adult borers than other shades. Dark brown, russet brown, and light brown did not differ significantly, whereas saddle brown traps were the least attractive.

Relative Effects of Visual and Olfactory Cues Mahogany brown ground traps baited with pheromone lures caught significantly more adults than those without ($F=33.32$, $df=1$, $P<0.001$; Fig. 6).

Effect of Trap Shading Pheromone-baited mahogany brown ground traps positioned in the shade of banana plants caught more adults than did similar traps placed in sunlight ($F=9.27$, $df=1$, $P<0.05$; Fig. 7). There was no significant difference in the color measurements of the mahogany brown traps placed in the sun (L=34.4, a=4.2, b=4.4, hue angle=46.7, and chroma=6.1) and shade (L=34.5, a=4.0, b=4.3, hue angle=46.5, and chroma=5.9). Similarly, only a marginal difference (<1°C) was observed among the temperatures of the different traps placed in both locations.

Table 2 Color measurements of the different shades of brown tested in the trapping of *C. sordidus* in Guam

Trap color	L*	a*	b*	Chroma (C)	Hue angle (h°)
Dark brown	35.26±0.18	3.98±0.03	3.94±0.02	5.60±0.03	44.66±0.06
Mahogany brown	35.91±0.01	5.44±0.02	4.35±0.03	6.97±0.03	38.65±0.13
Russet brown	38.99±0.03	11.37±0.05	9.00±0.01	14.51±0.03	38.37±0.07
Saddle brown	48.37±0.01	9.25±0.06	20.62±0.05	22.60±0.03	65.83±0.10
Light brown	61.13±0.03	4.50±0.02	21.87±0.02	22.33±0.01	78.38±0.03

Data represent means of three observations

*L** indicates lightness of the color and it runs through the center of the color chart, where 100 at the top represents white and zero at the bottom represents black, *a** runs left to right on the color chart, and indicates a red shade when greater than zero (positive) and a green shade when lower than zero (negative), *b** runs vertically through the color chart, and indicates a yellow shade when positive and a blue shade when negative

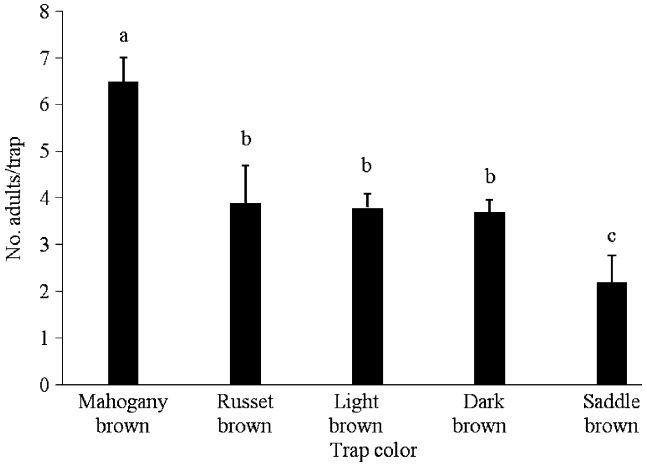

Fig. 5 Mean (± SE) numbers of adult *Cosmopolites sordidus* caught in pheromone-baited ground traps of different shades of brown. *Different lower-case letters* indicate significant differences between treatments (one-way ANOVA using Poisson model, Least Square Means, $P<0.001$)

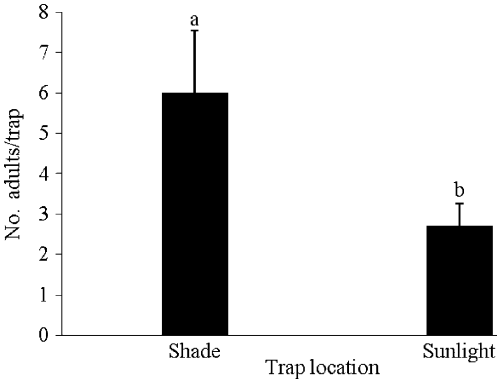

Fig. 7 Mean (± SE) numbers of adult *Cosmopolites sordidus* caught in pheromone-baited ground traps placed in shade and in sunlight. *Different lower-case letters* indicate significant differences between treatments (one-way ANOVA using Poisson model, Least Square Means, $P<0.05$)

Discussion

Pseudostem trapping was recommended for reducing populations of the banana borer (Gold et al. 2002; Koppenhöffer et al. 1994) but proved impractical and labor intensive (Gold et al. 2002). In Uganda, use of baited pitfall traps caught up to 18 times as many adults as pseudostem traps probably because they lasted longer (1 month vs. 3–7 days, Gold et al. 2001). While there have been several reports of successful trapping of banana borers with pheromone lures (Ndiege et al. 1996; Jayaraman et al. 1997; Alpizar et al. 1999), pheromone traps were not effective in reducing *C. sordidus* populations in Cameroon (Messiaen 2001). Tinzaara et al. (2005) failed to catch >3% of adults at either low or high trap density, and there was no reduction in damage to the plants (Tinzaara et al. 2005). Moreover, our recent studies indicate that, although ramp traps baited with Cosmolure are useful for monitoring *C. sordidus*, they do not reduce corm damage at several locations in Guam (G. V. P. Reddy, personal observation).

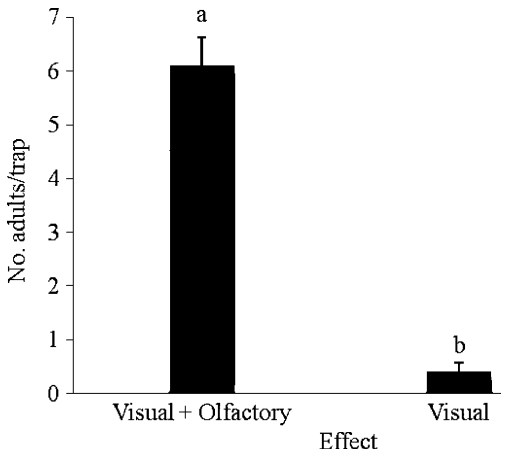

Fig. 6 Mean (± SE) numbers of adult *Cosmopolites sordidus* caught in mahogany-brown ground traps with and without pheromone lures. *Different lower-case letters* indicate significant differences between treatments (one-way ANOVA using Poisson model, Least Square Means, $P<0.001$)

Trap type is known to influence adult catches of many insects (Wyatt 1998; Reddy et al. 2005), yet no systematic study of trap features for more effective trapping of the banana borer had been conducted previously. Clearly, ground traps are superior to both ramp and pitfall traps under field conditions, as well as being cheaper and easier to use (Reddy 2007). Previous studies have shown that efficacy of traps for beetles can be strongly affected by their position (Lanier et al. 1976), color (Lanier et al. 1976; Ladd and Klein 1986; Smart et al. 1997), height above ground (Cuthbert and Peacock 1975), and surface texture (Lie and Bakke 1981), but few reports have been published on the effect of trap size. Tilden et al. (1979) found that larger traps caught significantly more western pine beetles *Dendroctonus brevicomis* than smaller traps. The same was reported for the European elm bark beetle, *Scolytus multistriatus* (Lanier et al. 1976), but not for the old-house borer, *Hylotrupes bajulus* (Reddy 2007).Trap diameter affected catches of spiders and ants (Brennan et al. 1999; Borgelt and New 2005), so it was surprising to find no difference between the three larger traps tested.

Some insects use visual pheromone cues to find a mate (Thornhill and Alcock 1983; Reddy et al. 2002), and these may act in combination (Carlton and Cardé 1990; Fukaya et al. 2004; Reddy and Guerrero 2004). Color has been shown to influence the efficacy of pheromone traps, and our results for the banana weevil show that not only is brown preferred

over the other colors tested but also that mahogany is preferred to other shades of brown. The borer's preference for mahogany brown did not obviate the need for a chemical attractant; however, pheromone-baited traps caught significantly higher numbers of adults than unbaited ones.

Finally, we studied the effect of shading on trap catches as placement is an important factor affecting trap efficacy (Blackmer et al. 2008). Although few reports address the effect of shade or sunlight on trap catches, our results are consistent with those of Arbogast et al. (2007), who reported a marked preference of the small hive beetle, *Aethina tumida* (Murray) (Coleoptera: Nitidulidae), for shaded traps over those placed in sunshine. As there were no significant differences in the temperature of traps placed in the shade or in the sun, it is unlikely that differences in trap catch were related to differential emission rates of the pheromone. Furthermore, as we recorded no differences in either the hue angle or chroma of the traps in sun and shade, it would appear that the differences were not related directly to the color properties of the traps, although this needs to be investigated. The most likely explanation relates to a behavioral preference for shade, possibly because beetles are subject to desiccation in sunlight.

In summary, we have shown that 40×25-cm mahogany brown ground traps baited with pheromone lures and placed in shaded areas of the banana plantation are an efficient tool for catching adult male and female banana borers. Although our experiments were conducted on a small Pacific island, we expect the new trap design to be useful for monitoring and mass trapping of this important pest in larger banana plantations in the mainland.

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Detection and Discrimination of Mixed Odor Strands in Overlapping Plumes Using an Insect-Antenna-Based Chemosensor System

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Abstract Olfactory signals, a major means of communication in insects, travel in the form of turbulent odor plumes. In terrestrial environments, an odor blend emitted from a single point source exists in every strand of the plume, whereas, in confluent plumes from two different odor sources, the strands have some chance of being coincident and comprising a new third odor in those strands. Insects have the ability to detect and interpret necessary olfactory information from individual filamentous odor strands in complex multifilament odor plumes. However, behaviorists have had no way to measure the stimulus situations they are presenting to their temporally acute insect subjects when performing Y-tube olfactometer or confluent pheromone plume wind tunnel assays. We have successfully measured the degree of plume-strand mixing in confluent plumes in a wind tunnel by using a multichannel insect-antenna-based chemosensor. A PC-based computer algorithm to analyze antennal signals from the probe portion of the system performed real-time signal processing and, following a short training session, classified individual odorant/mixture strands at sub-second temporal resolution and a few tens of millimeters of spatial resolution. In our studies, the chemosensor classified a higher frequency of strands of two different odorants emitted from two closely spaced filter papers as being "mixed" when the sources were located only 1 or 2 cm apart than when the sources were 5 or 10 cm apart. These experiments demonstrate the chemosensor's potential to be used for measuring odor stimulus situations in more complex multiple-plume environments.

Keywords Electroantennogram · Insect antennae · Odor detection · Odor strand · Odor plume · Tissue-based chemosensor · Volatile detection · Odor strand detection · Odor strand discrimination

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Introduction

Insects have demonstrated in behavioral experiments that use experimentally pulsed pheromone puffs that they are capable of discriminating pheromone plume strands of incompletely mixed components that are separated by only 1 mm from each other or equivalently by 1/1,000 of a second (Baker et al. 1998). However, researchers involved in presenting odors to insects that emanate from natural point source plumes in more complex contexts have not had the ability to measure the sub-second odor stimulus conditions against background odor plumes that may be experienced by insects in determining their behavioral responses. The ability to measure on a sub-second basis the degree of mixing of, for instance, plume strands of plant volatiles with those of synthetic or natural pheromone components or alternatively two different species' pheromone plumes would provide a quantitative basis for understanding olfactory temporal acuity that affects behavioral responses. The high time resolution of insect antennae to various odorants and the well-established signal recording technique from insect antennae such as electroantennogram (EAG) and

single-cell recording previously had given researchers the idea of using insect antennae as olfactory chemosensors to detect various volatile compounds of interest and ascertain fine-scale plume structure features (Baker and Haynes 1989; Sauer et al. 1992; Huotari and Mela 1996; Schöning et al. 1998; van der Pers and Minks 1998; Park et al. 2002).

In natural environments, point source odor plumes with their fine-scale plume-strand structures do not exist in solitude. The strands of a plume from one source can intermingle with those from another. The degree of confluent plume-strand coincidence or separation can have significant effects on behavior (Vickers and Baker 1992; Baker et al. 1998) or on olfactory receptor neuron (ORN) activity (Nikonov and Leal 2002; Ochieng and Baker 2002). We have had, to this point, however, no way of measuring the stimulus situations actually experienced by insects under various odor plume presentation regimes that affect their subsequent responses.

With single-antenna EAG recordings, odor discrimination cannot be performed due to the confounding effects of concentration and chemical composition. However, in recent studies, we showed that a four-channel insect-antenna-based chemosensor system that uses an array of slightly differentially tuned insect antennae, named the "Quadro-probe," could be used to discriminate various odors on a strand-by-strand basis (Park et al. 2002; Hetling et al. 2003; Myrick et al. 2005). The Quadro-probe has sub-second temporal resolution of individual odor strands (and a few millimeters of spatial resolution) that enables detailed odor-plume-strand structure and composition to be performed. A dedicated analysis software package (Hetling et al. 2003; Myrick et al. 2005) that can be trained to recognize EAG patterns across particular arrays of different insect antennae in response to different odors was also developed. The system has enabled the performance of real-time high-throughput odorant signal classification and monitoring. In the present study, the Quadro-probe system was used to investigate the degree of mixing of overlapping odor plume strands emitted from dual point sources, which provides a proof-of-concept foundation for investigating more complex multiple-point-source strand discrimination.

Our hypotheses were: first, that we should be able to differentiate between strands sheared from a single-source two-odorant mixture and those emitted from two closely spaced separate odorant sources; second, that there should be a greater degree of plume-strand coincidence downwind from two overlapping plumes of different odorants when the upwind sources are closer together than when the sources are more widely spaced. The ability to confirm or falsify these hypotheses and the ability to measure the degrees of plume-strand mixing have potential impact on various insect chemical ecology studies under natural conditions where plant volatiles may interact to influence olfaction and behavioral response to pheromones (Ochieng and Baker 2002) or where pheromone plumes of two species might interfere with males' behavioral responses (Witzgall and Priesner 1991; Liu and Haynes 1992; Vickers and Baker 1997; Nikonov and Leal 2002).

Methods and Materials

Insects One- to 3-day-old virgin male moths of the corn earworm, *Helicoverpa zea*, and the cabbage looper, *Trichoplusia ni* (Lepidoptera: Noctuidae), were used. Laboratory colonies of these species were maintained on artificial diet under controlled conditions (14:10 h L:D, 23°C, 60% relative humidity) in a rearing room. Male pupae were kept in separate containers, and emerged adults were collected daily and provided with 8% sucrose solution until use.

Test Chemicals Two compounds, (*Z*)-11-hexadecenal (Z11-16:Ald) and (*Z*)-7-dodecenyl acetate (Z7-12:Ac), were used in experiments 1 and 2. These compounds are the major sex pheromone components of *H. zea* and *T. ni*, respectively. Each compound (Bedoukian, >99% chemical and isomeric purity) was diluted in hexane to give a 1-μg/μl solution. A 1:1 mixture of these two compounds also was prepared at the same concentration. Filter papers (Whatman no. 1) cut to 8×40 mm were impregnated with the test solutions containing 10 μg of one of the three odors (Z11-16:Ald, Z7-12:Ac, or the 1:1 blend of the two compounds) and were then used to produce odor plumes in our wind tunnel. Each filter paper dispenser was used for less than 1 h and discarded. In experiment 3, a plant-related compound, citronellal (Acros Organics, 93% pure) at a dose of 100 μg per filter paper, was used as a second odorant to accompany the first odorant, Z11-16:Ald, at 10 μg per filter paper. A blend of these two compounds at these two doses emitted from a single filter paper comprised the blend, i.e., the third odor.

Wind Tunnel Experiments were carried out by using a Plexiglas® wind tunnel (1×1×2.5 m). Airflow was created by a large fan system, filtered with activated charcoal filter layers, and sent through a buffer area of three layers of fine mesh nets. An exhaust suction tube (50-cm diameter) was located at the downwind end of the tunnel to scavenge odor-bearing air from the plumes out of the wind tunnel room. The rest of the air from the tunnel was re-circulated through the room. Airflow rate was controlled by using a variable AC transformer to change electrical current for the fan system. A constant wind speed of 50 cm/s was maintained inside the tunnel, and the temperature was maintained at 23°C during experiments.

Experiment 1

Experimental Setup Two male *T. ni* and two male *H. zea* antennae were excised with micro-scissors from the heads and placed between stainless steel reference and recording electrodes to create the four input channels of the Quadro-probe. The bases of the four excised antennae were placed individually on each of the four reference electrodes, and their distal ends were brought into contact with the four different recording electrodes after clipping off a few terminal antennal segments. Electrical connections between the antennae and metal electrodes were secured with an electroconductive gel (Spectra 360, Parker Laboratories Inc., USA). EAG signals from the Quadro-probe were acquired with a headstage amplifier, amplified further with a main amplifier (Syntech, The Netherlands) and then processed and stored on a laptop PC. The Quadro-probe antennal preparation was placed within the time-averaged boundaries of the plume(s) in the middle of the tunnel at a downwind distance of 1.5 m during training sessions and at 1.5, 1.0, or 0.5 m downwind of the filter paper sources during various test situations. The filter paper dispensers were affixed to paper clips hung from metal stands 50 cm above the tunnel floor and 30 cm from the upwind end of the tunnel. Before the beginning of trials each day, a burning incense stick was placed at the upwind position where the stimulus dispenser was to be located. The plume of incense smoke was observed to ensure that the plume strands would be contacting the Quadro-probe antennal preparations.

Analysis Software A computer algorithm (Myrick et al. 2005) was used to acquire, process, and analyze four-channel EAG signals from the Quadro-probe; this system was run on a Labview-based platform (National Instruments, USA). The program carried out noise filtration, EAG signal identification and classification, and reporting of results. The classifier used a supervised *k*-nearest-neighbor (*k*-NN) procedure that was trained at the start of the experiment to recognize strands as being either Z7-12:Ac, Z11-16:Ald, or a 1:1 blend of the two compounds emitted from a single filter paper source. EAG "events" across the array were synchronized according to their occurrence within a 160-ms window. For instance, when a significant EAG depolarization (measured from trough to peak) having the greatest amplitude occurred on channel 1, the program created a 160-ms swath to look down onto the other channels to quantify the amplitudes of any other significant EAG events occurring on the other three channels.

Training and Classification with Single-Odor Plumes The session started with a period of training of the algorithm to three pheromone odor sources and clean air by subjecting the antennal array to plume strands from filter papers 1.5 m upwind that were dosed with 10 μg of test compounds (blank, Z11-16:Ald, Z7-12:Ac, and the 1:1 blend of Z11-16:Ald and Z7-12:Ac) for either a 20- or a 60-s period at a distance of 1.5 m downwind of the odorant source. These training periods were then used as criteria for odor strand classification during the subsequent test periods by the algorithm. We also tried a 60-s training period to see if classification accuracy would be improved. During the test periods, the antennal array was subjected to each test stimulus dispenser in random order for >120 s at 0.5, 1.0, and 1.5 m downwind of the odorant source, and the signals were digitized and stored. The number of replicates for each distance/stimulus combination is indicated in Table 1. For each test session, the signals were analyzed off-line by the computer algorithm for signal processing and odor classification.

Classification of Strands From Confluent Dual-Source Odor Plumes The degree of mixing of odor strands within overlapping plumes originating from two different closely positioned pheromone point sources was assessed by using the Quadro-probe system by detecting and classifying

Table 1 Comparison of performance of the EAG signal processing computer algorithm in positive signal identification (true positive) for odor strands of two sex pheromone components, (*Z*)-7-dodecenyl acetate and (*Z*)-11-hexadecenal, and their 1:1 mixture between two different training durations performed at 150 cm downwind and then tested at three different downwind distances

Distance (cm)	Stimuli	Percent correct recognition at training period of (mean±SE)		Number	*P* value
		20 s [total number of EAG signals]	60 s [total number of EAG signals]		
150	*Z*11-16:Ald	97.6±1.73 [113]	95.0±1.95 [127]	4	0.40
	*Z*7-12:Ac	100.0±0.00 [126]	100.0±0.00 [116]	4	N/A
	Mixture	89.5±4.75 [135]	92.6±1.53 [135]	4	0.39
100	*Z*11-16:Ald	92.8±5.58 [80]	93.8±3.27 [87]	3	0.65
	*Z*7-12:Ac	100.0±0.00 [79]	100.0±0.00 [114]	3	N/A
	Mixture	88.5±4.35 [100]	86.7±5.02 [79]	3	0.55
50	*Z*11-16:Ald	70.3±4.01 [161]	74.6±9.61 [138]	3	0.39
	*Z*7-12:Ac	100.0±0.00 [52]	97.0±3.03 [49]	3	0.30
	Mixture	100.0±0.00 [145]	98.1±1.20 [188]	3	0.08

Average number of EAG signals (depolarizations) in 20-s recording durations was 32.7±1.44 (mean±SE, N=84, range 7~73).

individual odor strands in the confluent plumes. Following the training session to the three separate odors from single sources, the same antennal preparation was used for the test sessions. The Z11-16:Ald and Z7-12:Ac odor sources were suspended on the rod with a 5-cm lateral separation between them at the upwind end. During this test period, signal acquisition and classification was performed for 120 s. Data were stored on a laptop PC for later analysis.

Experiments 2 and 3

Experimental Setup In the second and third experiments, live-moth preparations were used instead of excised antennae. Two male *T. ni* and two male *H. zea* were immobilized in tapered aerated plastic tubes and placed in a custom four-channel preamplifier (Fig. 1). With an electrolytically sharpened tungsten electrode inserted into the eye as a ground reference, the two antennae of each moth (with the antennal tips intact) were draped over the amplifier electrodes using Spectra 360 electroconductive gel to establish a connection to the amplifier.

Training Sessions Initial training sessions of approximately 100 s in plumes of each of three different odors, i.e., odorant 1 alone, odorant 2 alone, and a blend of odorants 1 and 2 at their initial dosages emitted from a single filter paper source, were conducted at the start of each experiment. EAG recordings were taken for an unpredetermined duration in the plume, but frequencies of classified odorant/odor "events" were standardized according to an events-per-second criterion.

Experiment 2 Once the training sessions had ceased, the Z11-16:Ald and Z7-12:Ac filter papers were suspended at the upwind end of the tunnel, beginning with a 1-cm lateral separation of the filter paper sources. After a period of sampling of approximately 100 s (ranging from 38 to 115 s) of strands from within a zone of confluence of the two plumes, the two sources were then separated by 2, then 5, and finally 10 cm. A second series of recordings was performed in reverse order, starting with the 10-cm source separation and working back to the 1-cm separation. Three experiments were performed, and because both increasing and decreasing the separation of the filter papers were known sources of potential variance, these experiments were broken into six blocks. Using Minitab ver. 14, two-way (distance of filter paper separation, block) analysis of variance (ANOVA) was performed on a general linear model of the strand frequencies of each odor. Tukey's pairwise comparisons were also performed on the model.

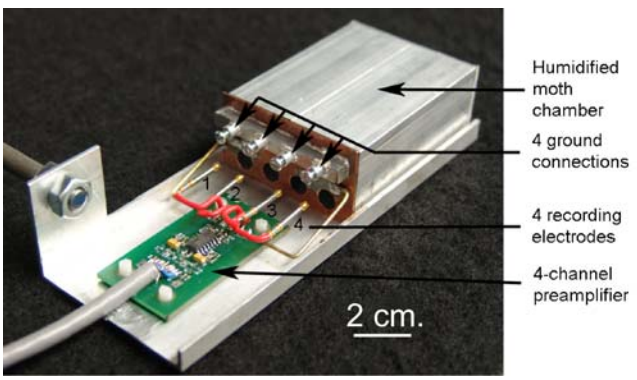

Fig. 1 Photograph of the whole-body preparation of the four-antenna Quadro-probe chemosensor probe using whole moths confined in plastic tubes within an aerated humidified aluminum chamber

Experiment 3 As in experiment 2, once the training sessions had ceased, the Z11-16:Ald and citronellal filter papers were suspended at the upwind end of the tunnel, beginning with a 1-cm lateral separation of the filter paper sources. After a period of sampling the odor strands within the confluent plumes, the two sources were separated by 2, then 5, and finally 10 cm, with data at all these distances being reported by the whole-body four-channel EAG preparation and stored on laptop PC for later analysis. Similar to experiment 2, a second series of recordings was performed in reverse order, starting with the 10-cm source separation and working back to the 1-cm separation. Two experiments were performed, and because both increasing and decreasing the separation of the filter papers were known sources of potential variance, these experiments were broken into four blocks. Using Minitab ver. 14, two-way (distance, block) ANOVA was performed on a general linear model of the strand frequencies of each odor. Tukey's pairwise comparisons also were performed on the model.

Classification of Odor Strands from Dual-Source Plumes In the second and third experiments that used live-moth preparations, the classifier made use of a Gaussian model for the probability density functions describing each odor. The preamplifier, seated in the whole-body apparatus, was connected to the recording system as described in Myrick et al. (2005). Data were filtered with a 2–15-Hz digital finite impulse response band pass filter prior to feature extraction. The feature type utilized in classification included time-synchronized EAG depolarization trough-to-peak voltages (TPV) only. When a depolarization occurred, trough-to-peak voltages (where a peak describes a *negative*-going depolarization) on different channels with (negative) peak times occurring within 160 ms were grouped to form a four-dimensional feature vector, called an event, with a feature corresponding to each channel. Missing data (when no peak was found on a channel) were filled with a value of 0. Any event (collection of four time-correlated trough-to-peak voltages) with no peaks larger than 100 μV was discarded as a simple strand detection procedure. The

trough-to-peak voltages in the remaining events were normalized by using the same constant so that the standard deviation of all the trough-to-peak values in the training set on all of the channels was unified. Note that this operation retains concentration information. The same value was used to keep noise levels relatively equal on each channel.

After collecting several hundred training events (100 s) for each odor, the probability densities of each odor were modeled by using a multidimensional Gaussian distribution. The Gaussian is able to model the unique correlation between the channels for each odor. The distribution was derived from a four-dimensional model, where variation along the covariance matrix eigenvector with the largest eigenvalue was ignored, so that the dimensionality of the distribution was reduced by 1. The component of any feature vector that points along this eigenvector is considered to be a measure of signal strength. The multidimensional Gaussian density (MDG) may be expressed by the following equation (Theodoridis and Koutroumbas 1999).

$$f(x) = \frac{1}{\sqrt{(2\pi)^d |\sum|}} \exp\left(-1/2(\mathbf{x}-\mu)^T \sum^{-1} (\mathbf{x}-\mu)\right)$$

where $\mathbf{x}$ is a vector of d (four) elements containing the feature values of an event (i.e., trough-to-peak measurements from multiple antennae); $\mathbf{\Sigma}$ is the sample covariance matrix of the trough-to-peak measurements for the odor, and $\boldsymbol{\mu}$ is the vector containing the feature means. The exponent of the MDG may be written as a summation in terms of the eigenvectors and eigenvalues of the covariance matrix. The sample covariance matrix may be expressed in terms of the eigenvectors and eigenvalues, known as spectral decomposition, or eigendecomposition, making its inverse simple to calculate (Johnson and Wichern 1992). We write it this way to explicitly remove dependence of the probability density in the direction of the eigenvector with the largest eigenvalue, so that the probability density near the mean is extrapolated through the origin. This estimates the density near the origin, where background and noise reside, since it cannot be measured directly. The problem of not having the ability to train on pure odor (that is only odor + background or background is available during training for an odor) is known as the imperfect teacher problem (Krishnan 2001) for mixture densities. It also makes the classification result independent of any prior knowledge of the distribution of signal strength for a particular odor. Let the index of the largest eigenvector and corresponding eigenvalue be $n$max. The reduced dimensionality MDG may be written

$$f(x) = \frac{1}{\sqrt{(2\pi)^{d-1} \prod_{n=1, n\neq n\max}^{d} \lambda_n}} \exp\left(-\frac{1}{2} \sum_{n=1, n\neq n\max}^{d} \frac{(\mathbf{e}_n \bullet (\mathbf{x}-\mu))^2}{\lambda_n}\right)$$

where $\mathbf{e}_n$ and λ_n are the nth eigenvector and eigenvalue of the sample covariance matrix for a particular odor, respectively. An example that illustrates the odor density functions superimposed on training data for two channels (rather than four channels) is shown in Fig. 2. Class membership of a new normalized vector (event) is then determined by using Bayesian inference assuming each odorant has an equal prior probability. Details regarding pattern recognition techniques can be found in the following reference (Theodoridis and Koutroumbas 1999).

Quadro-probe Longevity and Odor Classification Longevity After conducting the plume-strand-mixing trials in experiments 2 and 3, each Quadro-probe preparation was maintained and tested at several time intervals, ending at 24 h after the initial wind tunnel tests. For the responses of the antennal array to strands from a plume of each of the odorants 1 and 2 and also to strands of the mixture of the two odorants on a single filter paper (odor 3), the subsequent analyses of odor strand classification were carried out and compared as scatter diagrams to those that were made by the system during the first hour of recording.

Maximum EAG Longevity To determine the maximum longevity of antennal responsiveness using another version of the whole-body preparation, a male *T. ni* moth was restrained on a Plasticine block with U-shaped thin copper wires. A fine-tip reference capillary glass electrode filled with 0.5-M KCl solution was inserted into a compound eye, and another capillary glass electrode filled with electro-conductive gel was brought into contact with antennal tip after cutting a few terminal antennal segments. The antenna was then placed in a charcoal-filtered humidified continu-

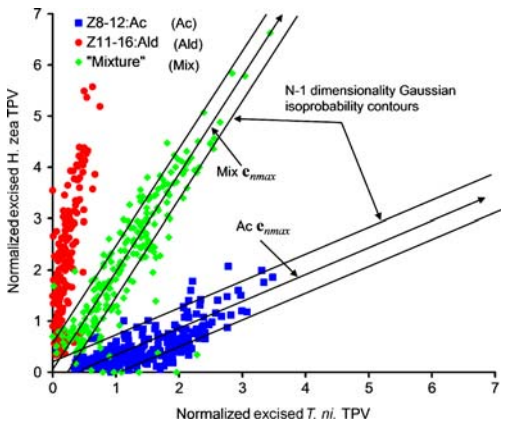

Fig. 2 Two-channel scatterplots of events obtained from four-channel simultaneous recordings of excised antennae from male *Trichoplusia ni* and male *Helicoverpa zea* antennae. These data were thresholded at 100 μV, as described in the text. Two-dimensional eigenvectors (purely illustrative) corresponding to the largest eigenvalues for Z8-12:Ac and mixture are shown. Isoprobability contours for these odors are drawn on either side of the eigenvectors

ous airflow (600 ml/min) at ~3 cm from the air outlet of a glass tube (8 mm ID). Stimuli were delivered by giving single 30-ms puffs (2-ml volume) of charcoal-filtered air through a large opening of a Pasteur pipette containing a filter paper strip (8×40 mm, Whatman no. 1) containing 0, 1, 10, or 100 ng of Z7-12:Ac, while the small opening of the Pasteur pipette was placed in the airflow through a small (3-mm diameter) opening in the glass airflow tube. EAG responses were measured at several different times each day until no EAG responses were observed. Three antennal preparations were tested; three consecutive stimuli were delivered at each measurement time.

Results

Experiment 1 In the wind tunnel, EAG responses to clean air (i.e., baseline activity) were usually lower than 50 μV at various airflow rates. Although some drift of baseline amplitude was observed in some antennae, it did not appear to significantly affect our chemosensor system performance. When two male *H. zea* antennae and two male *T. ni* antennae were used for the Quadro-probe and Z11-16:Ald, Z7-12:Ac, and 1:1 mixture of these two compounds were tested (all three odors emitted from single filter papers), *H. zea* antennae showed strong responses to Z11-16:Ald and the mixture (Fig. 3 Ald, mixture), and *T. ni* antennae showed strong responses to Z7-12:Ac and the mixture (Fig. 3 Ac, mixture).

The EAG depolarizations in response to odor strands from the two antennae of the same species were synchronized (Fig. 2) and exhibited consistent response proportionality over a wide range of pheromone strand flux strengths (Fig. 4a). The ratio of EAG amplitudes in response to the two-component mixture of *H. zea* antennae and *T. ni* antennae also was maintained regardless of the flux (amplitude of the EAGs) of the mixture odor strands (Fig. 4b). The Quadro-probe system thus was able to classify and discriminate the strands of these three different

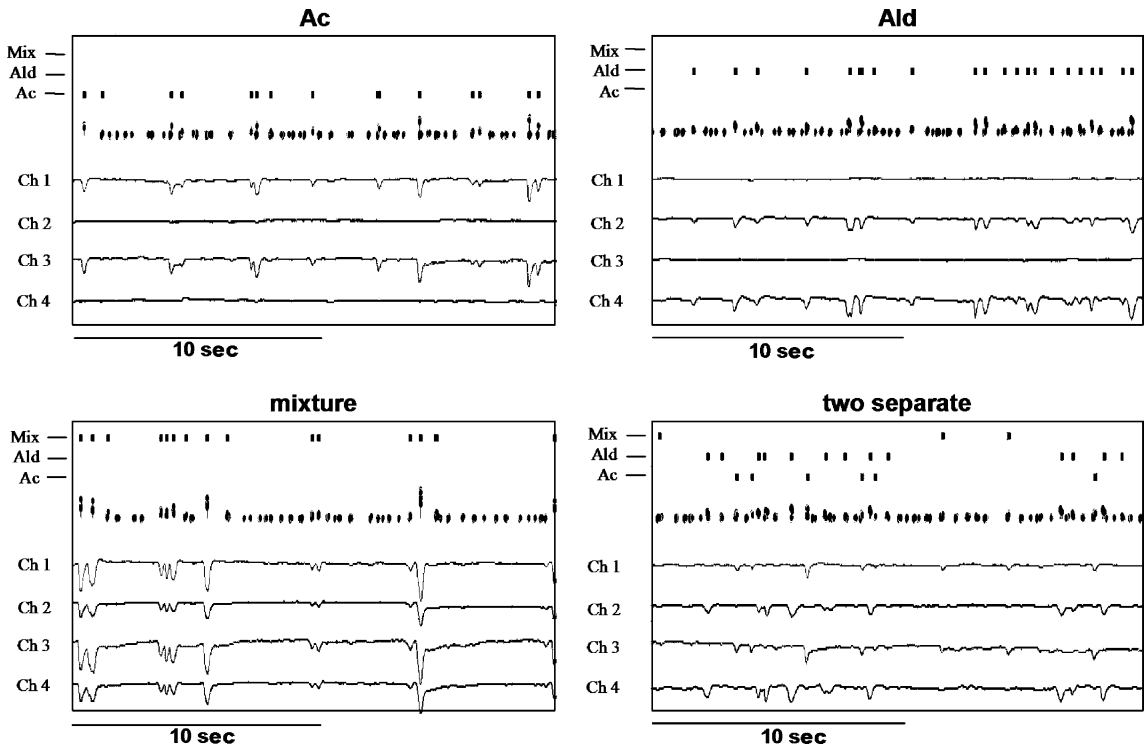

Fig. 3 Recordings from the four-antenna chemosensor system in different odor plumes in the wind tunnel. *Horizontal scale bar* 10 s. *Vertical scale* is 4 mV separation between channels. Each depolarizing peak (downward deflections in the four continuous-time traces) was an EAG response to contact with an individual odor filament. Four different moth antennae were used for the four-channel chemosensor probe. Ch 1 and ch 3 are male *T. ni*. Ch 2 and 4 are male *H. zea*. The probe was placed 1.5 m downwind from the filter paper test odor dispensers. Wind velocity was maintained at an average of 50 cm/s. The system was "trained" first to a plume of "Ac" (*Z*7-12:Ac), then to "Ald" (*Z*11-16:Ald), and finally to a mixture of the Ac and Ald placed on a single source ("mixture"). These sources were then used in the test situations illustrated here. The two separate sources of Ac and Ald were placed 5 cm crosswind from each other. *Lollipop-type symbols* along the *fourth line* from the *top* indicate depolarization "events" on individual channels that were detected by the system, and the *height* of each *oval* indicates the amplitude of each depolarization; channels are not indicated. Among these signals, those over an operator-determined threshold level were further analyzed and classified as Ac (*lowest row* of *squares*), Ald (*middle row* of *squares*), or a mixture of the two (*upper row* of *squares*)

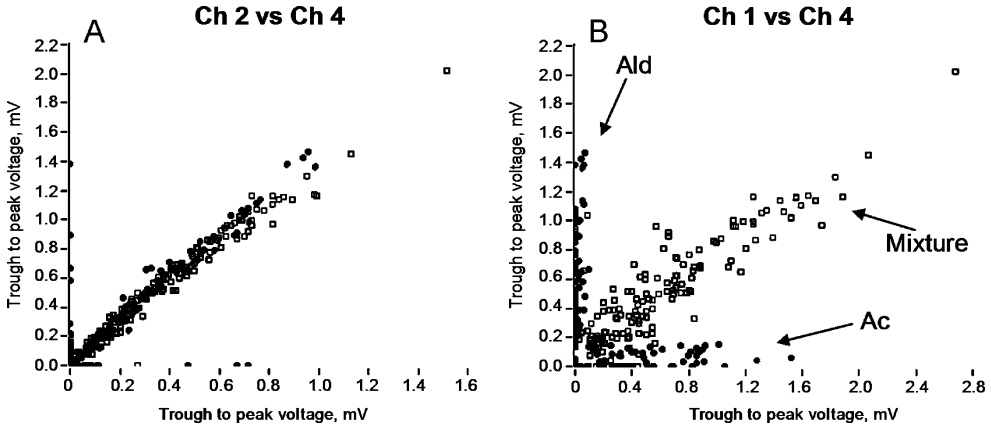

Fig. 4 The response amplitude relationships between two different antennae in the array, illustrating the profiles generated between two *T. ni* antennae (**A**, channel 2 vs. channel 4) and one *T. ni* and one *H. zea* antenna (**B**, channel 1 vs. channel 4) when exposed to strands from single filter paper sources of the Ac, Ald, or mixture of Ac + Ald, respectively. Regardless of EAG depolarization amplitude, same-species antennae (**A**) show a high-fidelity linear relationship regardless of the odor strand flux they are sampling. Two different species' antennae (**B**) also exhibit high-fidelity linear relationships regardless of the strand flux strength, but now these are exhibited in three distinct odor spaces that are characteristic of the three odors. Multidimensional *k*-nearest-neighbor analysis using clusters from all combinations of the four channels was the basic criterion used by the algorithm for odor classification

pheromone odors over a wide range of EAG amplitude flux with high accuracy (Table 1).

There was no significant difference in odorant recognition performance of the algorithm whether the training session was 20 or 60 s long (Table 1; there were no significant differences in odorant recognition performance when the tests were conducted at distances closer to the source than the 1.5 m training distance, except for Z11-16:Ald being less often correctly identified at 0.5 m than at the two longer distances). *P* values obtained from 2×2 (correct/incorrect × 20/60 s) chi-squared tests are listed in Table 1. Over all trials and all three test distances from the source, this experiment showed 98%, 86%, and 86% correct odor identification rates for Z11-16:Ald, Z7-12:Ac, and their mixture on a single source, respectively. When two different point odor sources (Z11-16:Ald and Z7-12:Ac) were placed 5 cm laterally apart at the usual upwind position in the wind tunnel, the Quadro-probe chemosensor system identified the individual strands from the confluent plumes of the two odors, plus instances in which the strands coincided within the 160 ms time window, registering a classification as the mixture (Table 2).

Experiment 2 The degree of synchronous arrival of plume strands arriving at the probe within the 160 ms time window conforms to the prediction of a higher frequency of occurrence of mixed strands when the sources are very close together. When the two pheromone odorant sources were separated laterally by 1 or 2 cm, there was a greater number of strands per second contacting the four-antenna probe downwind that were classified as "mixture" than when the sources were separated by 5 or 10 cm (Fig. 5). A mean of 1.75 strands per second was classified as being mixed at 1-cm filter paper separation compared to 0.69 strands per second at 5-cm separation and 0.34 strands per second at 10 cm separation that were classified as being mixed (Fig. 5).

The frequency of mixed strands (1.75 per second) as a proportion of the frequency of all three types of strands classified by the system (3.39 per second) was 0.52 at the 1-cm filter paper spacing, whereas it was 0.12 at the 10-cm spacing (0.34 per second mixed versus 2.93 per second total strands (Fig. 5); at the 5-cm spacing, this proportion was 0.18 (0.69 per second mixed versus 3.92 per second total strands). At the 2-cm filter paper spacing, there was

Table 2 Identification performance of the four-antenna chemosensor system for odor strands of two sex pheromone components, (*Z*)-7-dodecenyl acetate (Ac) and (*Z*)-11-hexadecenal (Ald), in a wind tunnel

Odor stimuli	Percent ratio identified as (mean±SE)			Total no. of EAG signals	Number
	Ac	Ald	Mixture		
Ac	86.1±6.41	5.4±1.30	8.5±5.96	66	3
Ald	2.4±2.38	97.6±2.38	0.0±0.00	75	3
Mixture of Ac and Ald on single source	2.6±1.29	11.4±5.56	86.0±5.18	149	5
Ac and Ald on two separate sources placed laterally 5 cm apart	38.7±7.75	42.3±6.22	19.0±4.21	509	13

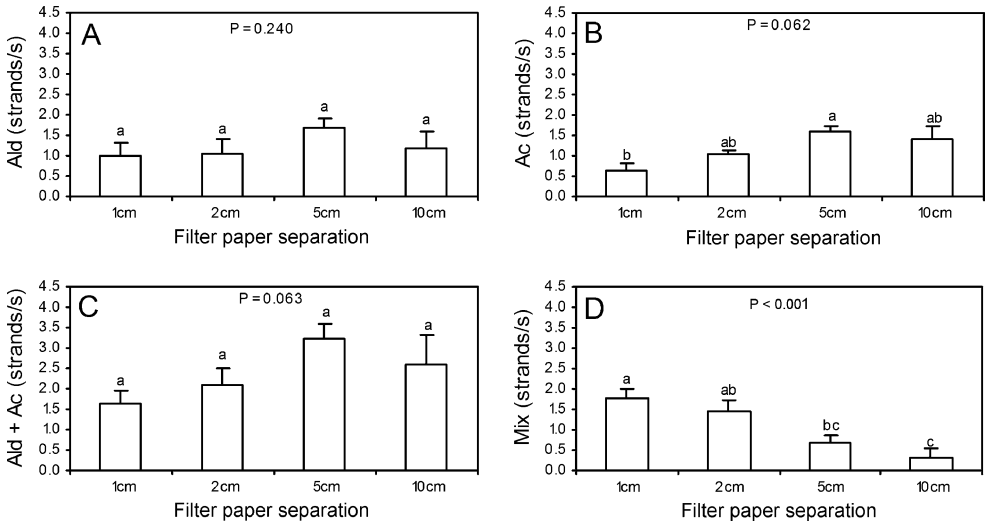

Fig. 5 Histograms (mean±SE) of the response frequencies, per second, of classified odor strands using a whole-body Quadro-probe preparation placed 1.5 m downwind of two closely spaced filter paper odor sources in the wind tunnel. Trials were conducted by using a filter paper emitting Z11-16:Ald and a second filter paper emitting Z7-12:Ac that were separated by 1, 2, 5, and 10 cm crosswind at the upwind end of the tunnel. EAG responses from the probe were classified as **a** Z11-16:Ald strands (Ald), **b** Z7-12:Ac strands (Ac), or else **d** "mixed" strands arriving coincidentally on the four-antenna array (mix). The sum of single strands classified as either Ald or Ac is shown in **c**. The more widely the filter papers were spaced, the less likely were plume strands to be judged as "mixed" (**d**) by the Quadro-probe system. A multidimensional Gaussian distribution was the basis for the classifier algorithm. N=3 antennal preparations, with two blocks each. Two-way (distance, block) ANOVA was performed on a general linear model of the strand frequencies of each odor, yielding P values of 0.240, 0.062, 0.063, and <0.001 for Ald, Ac, Ald + Ac, and mix, respectively. Tukey's pairwise comparisons resulted in 95% confidence groupings indicated by *lettering* located *above* each *bar*; means having no letters in common are significantly different at $P \leq 0.05$. The mean number of strands classified (all three odors) in each of the replicates in this experiment was 257 for the 1-cm filter paper separation, 276 for 2 cm, 262 for 5 cm, and 138 for 10-cm separation. Total strand frequency (all three odors) diminished as spacing increased, going from 3.84, 3.52, and 3.94 to 2.93 per second at 1-, 2-, 5-, and 10-cm filter paper separation, respectively

nearly as high a proportion (0.41) of mixed strands as at the 1-cm spacing (0.52). At 2 cm, the frequency was 1.44 per second mixed versus 3.52 per second total strands (Fig. 5). Thus, the asynchronous arrival of pheromone odor strands from two different pheromone sources can be detected and discriminated by the Quadro-probe system and varies according to odor source separation distance.

Experiment 3 Although the proof-of-concept ability of the insect-antenna-based probe plus software to discriminate odor strands separated in time downwind was demonstrated in experiment 2, we conducted a third study involving citronellal, a plant-volatile-based compound that provided a non-pheromonal background-noise plume against the signal from a pheromone plume. This situation that might occur when a female moth emits pheromone against a background of point source floral odors or induced plant volatiles from leaves.

Following training to citronellal, Z11-16:Ald, and a mixture of the two emitted from a single filter paper, the whole-moth-body 4-antenna system classified a greater frequency of odor strands as "mixture" when the plant- and pheromone-based sources were separated by 1 or 2 cm than when they were more widely separated by 5 or 10 cm (Fig. 6). A mean of 1.62 strands per second were classified as being mixed at the 1-cm filter paper separation compared to 0.56 and 0.28 strands per second that were classified as being mixed at the 5- and 10-cm separations, respectively.

The mixed strand frequency (1.62 per second) as a proportion of the total strand frequency of all three odors (3.45 per second) was 0.47 at the 1-cm filter paper spacing, whereas it was 0.10 at the 10-cm spacing (0.28 per second mixed strands versus 2.83 per second total strands (Fig. 6). At the 5-cm spacing, this proportion was 0.13 (0.56 per second mixed strands versus 4.24 per second total strands). At the 2-cm filter paper spacing, the proportion of mixed strands was 0.24 (0.87 per second mixed versus 3.56 per second total). Thus, the asynchronous arrival of plant odor strands and pheromone odor strands was detected and discriminated by the Quadro-probe system. As in experiment 2 that used two pheromone sources, the degree of synchronous arrival of plant odor strands and pheromone strands arriving at the probe within the 160-ms time window conforms to the prediction of a higher frequency of occurrence of mixed strands when the sources are very close together.

Whole-Body Quadro-probe Antennal Longevity The responsiveness of the Quadro-probe system array was preserved over 24 h (Fig. 7). The scatter diagrams show normalized training data (TPVs) obtained during three trials. Information obtained from two antennae are shown

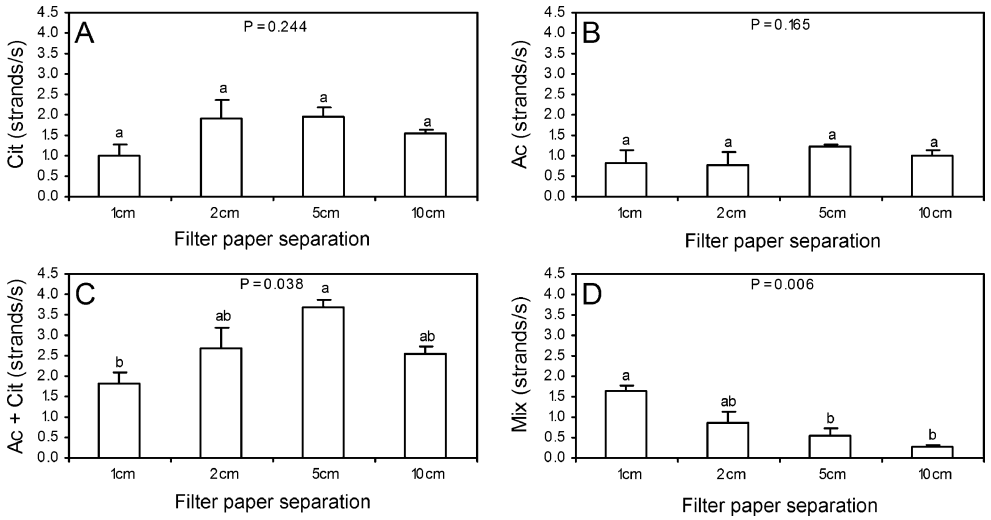

Fig. 6 Histograms (mean±SE) of the response frequencies, per second, of classified odor strands using a whole-body Quadro-probe preparation placed 1.5 m downwind of two closely spaced filter paper odor sources in the wind tunnel. Trials were conducted by using a filter paper emitting citronellal and a second filter paper emitting Z11-16:Ald that were separated by 1, 2, 5, and 10 cm crosswind at the upwind end of the tunnel. EAG responses from the probe were classified as **a** citronellal strands (Cit), **b** Z11-16:Ald strands (Ald), or else **d** "mixed" strands arriving coincidentally on the four-antenna array (mix). The sum of single strands classified as either Ald or Cit is shown in **c**. The more widely the filter papers were spaced, the less likely were plume strands to be judged as "mixed" (**d**) by the Quadro-probe system. A multidimensional Gaussian distribution was the basis for the classifier algorithm. N=2 antennal preparations, with two blocks each. Two-way (distance, block) ANOVA was performed on a general linear model of the strand frequencies of each odor, yielding P values of 0.244, 0.165, 0.038, and 0.006 for Cit, Ac, Cit + Ac, and mix, respectively. Tukey's pairwise comparisons resulted in 95% confidence groupings indicated by *lettering* located *above* each *bar*; means having no letters in common are significantly different at $P \leq 0.05$. The mean number of strands classified (all three odors) in each of the replicates in this experiment was 383 for the 1-cm filter paper separation, 418 for 2 cm, 459 for 5 cm, and 414 for 10-cm separation. Total strand frequency (all three odors) diminished as spacing increased, going from 3.45, 3.54, and 3.80 to 2.83 per second at 1-, 2-, 5-, and 10-cm filter paper separation, respectively

in each panel, after strand detection. Training data obtained at 0 and 24 h for trial 1 are shown in Fig. 7a, b, respectively. Similarly, training data collected at 0 and 24 h are shown in Fig. 7c, d for trial 2 and Fig. 7e, f for trial 3. Only two channels were recorded in trial 1, while four (two of each species) were recorded (only two shown) in trials 2 and 3. The reason trial 1 has less data near the origin is that there is less noise immunity with only two channels. To show that the live preparations are effective for at least 24 h, we measured the performance of the classification system in terms of error rate for each trial at 0 and 24 h. We used the empirical error rate measure on re-substituted training data with the two-stage classification method outlined in Myrick et al. (2008). Briefly, this method uses a detection procedure (stage 1) to separate "significant" (compared to baseline activity) strands from "insignificant" strands prior to classification using a k-NN method (stage 2). Stage 2 allows for the rejection of ambiguous responses, which was not utilized here (ambiguity reject parameter of 0.0). The percent correct classification for each odor is shown in each panel with the order being (clockwise from the upper left) Z7-12:Ac, mixture, and Z11-16:Ald (Fig. 7).

Single Antenna Longevity When a different form of the whole-body preparation was employed and monitored by using single *T. ni* antennae, EAG responsiveness to different amounts of the major sex pheromone component, Z7-12:Ac, lasted for longer than 60 h (Fig. 8). The EAG responsiveness of the whole-body preparation was maintained without reduced amplitude compared to the first hour for greater than 50 h, with a relatively steep drop-off thereafter.

Discussion

Whole antennae have been used for EAGs that report the peak-to-trough fluctuations in odor strand flux that occur on a several Hertz basis in natural odor plumes (Vickers and Baker 1994; Baker et al. 1998; Vickers et al. 2001; Bau et al. 2002, 2005; Vickers 2006). Experimentally pulsed odorant strands showed that there is a temporal resolution of EAGs that is much higher than this: up to 25 Hz in *Bombyx mori*, 5 Hz for *Lymantria dispar* (Bau et al. 2005), 33 Hz for *Spodoptera exigua* and *Cadra cautella*, and up to 25 Hz in *Pectinophora gossypiella* (Bau et al. 2002). Characterization of the antenna as an information transducer revealed a simple linear first-order low-pass filter with a −3-dB bandwidth of approximately 10 Hz (*S. exigua*) and information carrying capacity of up to 37 bits/s. (Justice et al. 2005)

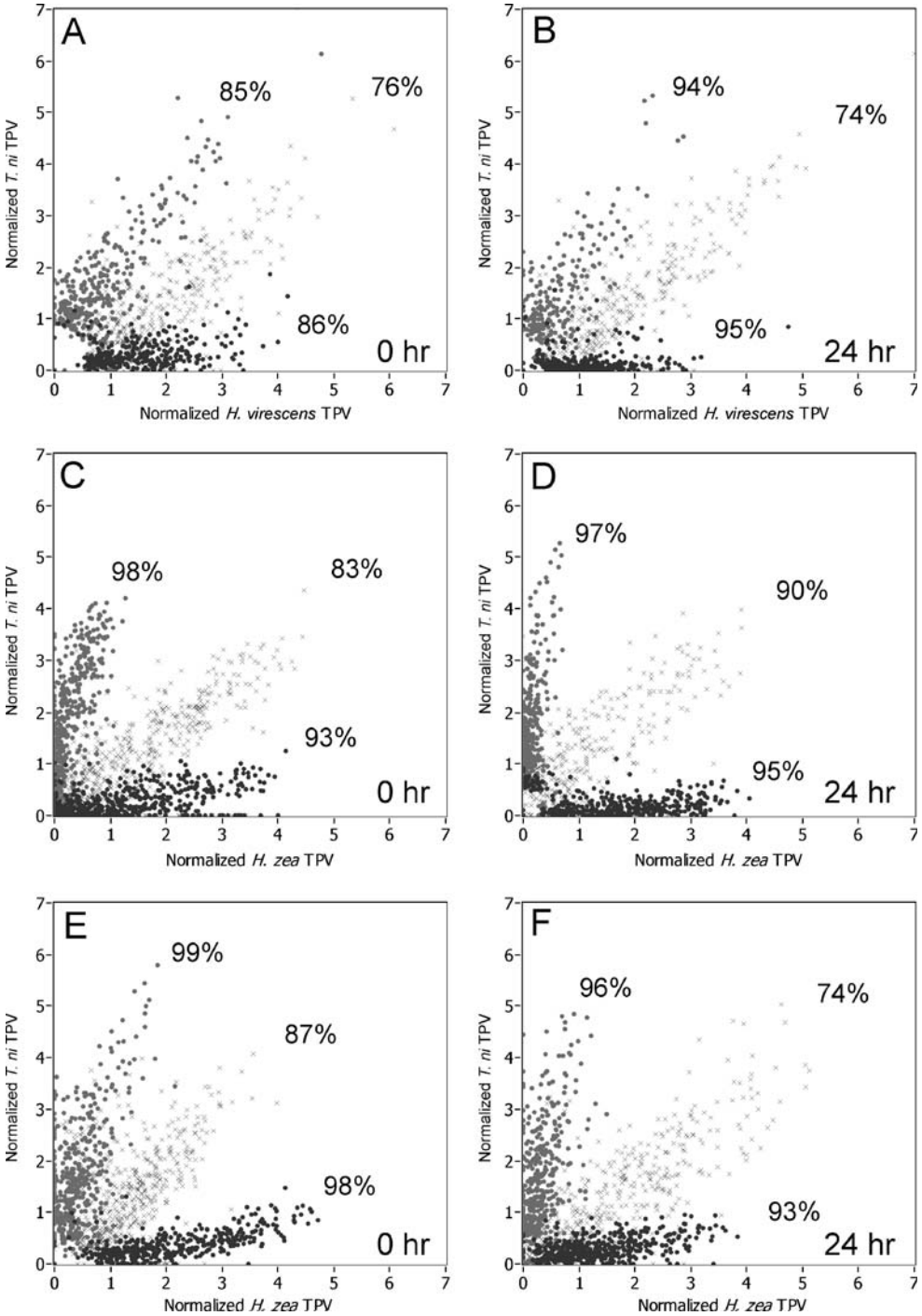

Fig. 7 Training data and classification accuracies (in percent) obtained from the Quadro-probe whole-body preparations over 24 h in response to strands of Z11-16:Ald, Z7-12:Ac, and the mixture of the two on the same filter papers presented as single-point-source odor plumes in the wind tunnel. *TPV* is trough-to-peak voltage. The four-antenna preparation was placed 1.5 m downwind of the filter paper source. Each *shaded symbol* is a classified response to an individual odor strand. *Black* = "aldehyde," *dark gray* = "acetate," *X symbol* = mixture. Highest depolarization amplitudes in response to odor strands were approximately 2 mV. **a**, **b** Trial 1 training data at 0 and 24 h, respectively. **c**, **d** Trial 2 training data at 0 and 24 h, respectively. **e**, **f** Trial 3 training data at 0 and 24 h, respectively. Each trial is a different whole-moth preparation. Error rates obtained (see text) for each re-substituted set of training data are converted into percent correct and labeled near the data corresponding to each odor; the percentages correspond to Ac, mix, and Ald in *clockwise* direction on each panel. Only one *T. ni* and one *H. zea*/*virescens* whole-moth EAG channel could be used for each two-dimensional depiction. N=3 replicates (trials)

Despite these previous successes of using single-antenna EAGs, quantification of the degree of odor strand mixing within overlapping odor plumes has not been possible to date because of an inability not only to detect but to classify on a strand-by-strand basis the odor strands in plumes comprised of different odorants. The present study shows that we can resolve incompletely mixed intertwined strands of odor within confluent plumes and classify their composition.

Our results show that two confluent plumes that on a time-averaged basis would appear to be perfectly mixed are in fact incompletely mixed when examined with the resolution provided by the insect antennal flux-detector tissue. We should now be better able to quantitatively relate an insect's behavior in response to complex odor plume interactions to the degree of mixing of plume strands that the insect experiences downwind. Experiments can be performed now not only with experimental pulses but also

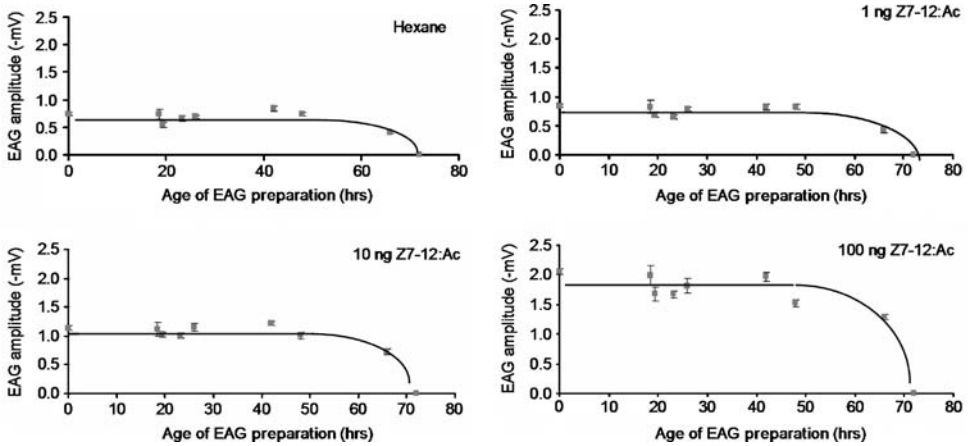

Fig. 8 EAG responsiveness of a whole-body preparation of male *T. ni* to different doses of Z7-12:Ac acetate over time (Mean± SE, $N \approx 7–45$)

with more natural point source plumes that overlap to different degrees.

The ability of the Quadro-probe to quantify incompletely mixed strands of odors that emanate from two or more different point sources is an apparent advantage of the insect antenna-based chemosensor over many other existing biomimetic artificial nose systems. In our experiments, only a small percentage of strands from two confluent plumes separated by more than 5 cm at their source in the wind tunnel arrived closely enough in time that the system declared them to be a "mixture." Further improvements on the system's range of odor measurement abilities will, of course, improve its applicability to more varied semi-ochemical systems that involve different combinations of odor plumes. In addition, improvement of the temporal resolution of the analysis software by further reducing its current 160-ms time window for declaring strands to be mixed vs. separate will more closely mirror insect olfactory systems' temporal resolution abilities.

Insects have been shown in behavioral experiments to discriminate between two confluent odor plumes as opposed to a blend of the two odorants placed on a single source (Witzgall and Priesner 1991; Liu and Haynes 1992; Baker et al. 1998). In behavioral studies, *H. zea* was able to distinguish between experimentally generated odor strands separated by 0.001 s in time and 1 mm in space (Baker et al. 1998; Fadamiro et al. 1999). There is also evidence in scarab beetles that puffs of two different pheromone components that arrive on ORNs with less than 1-ms separation cause different levels of ORN stimulation than when they arrive simultaneously (Nikonov and Leal 2002). Thus, the resolving power for this olfactory feat, at least in part, appears to result from mixture interactions that occur "on-site" (Baker et al. 1998; Todd and Baker 1999) between intimately associated ORNs that are co-compartmentalized within individual sensory hairs, pegs, or plates.

One of the most fragile aspects of any insect-antenna-based chemosensor has been its longevity of responsiveness. This has typically been less than 1 h (van Giessen et al. 1994; Hardie et al. 1994; Visser et al. 1996; Visser and Piron 1997; Park et al. 2002). The limited lifetime of the preparation has been a limitation in various other studies that employ living tissues as olfactory chemosensors (Kuwana and Shimoyama 1998; Kuwana et al. 1999; Schroth et al. 1999; Schütz et al. 1999). However, we have shown in this study that the longevity of the Quadro-probe EAG preparations can be improved significantly by using the entire moth bodies (Park and Hardie 1998; Park et al. 2002). Indeed, when whole-body preparations were used instead of isolated antennae, EAG responsiveness was shown to last up to 50 h with no decline in amplitude, and the fidelity of classifying odorants and blends of odorants by the multiantenna system could last as long as at least 24 h without significant reduction of response.

The extremely high sensitivity of insects' olfactory systems has been well documented (Angioy et al. 2003). A recent comparison between the peripheral position of this insect system, the antennae, and one of the most sensitive commercial electronic noses, the Cyranose 320, showed that insects have one to two orders of magnitude better sensitivity than the electronic nose (Rains et al. 2004). A BioFET chemosensor was developed by connecting an insect antenna to a field-effect transistor, showing that it could detect 1-ppb concentration of *Z*-3-hexen-1-ol (Schöning et al. 2000). We can hope that fully non-tissue-based chemosensors (Schaller et al. 1998; Walt et al. 1998; Drake et al. 2003) can be developed that mimic some of the features of insect antennae, especially their high sensitivity flux-detection abilities that can be of use for detecting and classifying strands of different odors in overlapping plumes.

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Volatile Signals During Pregnancy: A Possible Chemical Basis for Mother–Infant Recognition

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Abstract Human pheromones play a role in regulating relationships and apparently influence partner choice and mother–infant recognition. We analyzed the chemical content of volatiles from sweat patch samples from the para-axillary and nipple–areola regions of women during pregnancy and after childbirth. Solid phase microextraction was used to extract the volatile compounds, which were then characterized and quantified by gas chromatography–mass spectrometry. During pregnancy, women developed a distinctive pattern of five volatile compounds common to the para-axillary and nipple–areola regions (1-dodecanol, 1-1′-oxybis octane, isocurcumenol, α-hexyl-cinnamic aldehyde, and isopropyl myristate). These compounds were absent outside pregnancy and had slightly different patterns in samples from the two body areas. Differentiation of the volatile patterns among pregnant women may help newborns to distinguish their own mothers.

Keywords Gas chromatography–mass spectrometry (GC–MS) · Human pheromones · Solid phase microextraction (SPME) · Sweat patches · Volatile compounds

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Introduction

In animal species, recognition between individuals is a prelude to any kind of further interaction. Recognition between mother and newborn is a fundamental behavioral interaction that is worthy of systematic investigation. The emotional relationship between a mother and her newborn begins with mutual recognition, which starts during gestation and continues through and after birth, augmented by body contact and lactation. Imprinting takes place through visual, auditory, and olfactory learning, which occurs very early during the so-called "critical period" (Chiarelli 2003). Consequently, from the beginning of pregnancy, olfaction seems to represent an Ariadne's thread that permits the infant after birth to find its mother.

Pheromones regulate reproductive behavior in many mammalian species. Once released in the environment, through urine or glandular secretions, these volatiles reach other individuals, signaling mating availability and strengthening ties between mother and offspring, as well as regulating social relationships (Scalfari 1994; Chiarelli 2001). In nonhuman vertebrates, pheromones are detected by a specific sensory apparatus, the vomeronasal system, composed of a peripheral organ located at the base of the nasal septum, the vomeronasal nerve, and a nerve center, the accessory olfactory bulb. The vomeronasal system is

separated and independent from the main olfactory system (Døving and Trotier 1998; Chiarelli 2003). It is triggered by a different class of volatiles called "odorants" and is present in many reptiles and in almost all mammals, but it is absent in fish and birds, even if they possess a main olfactory system.

Primates, long considered functionally nonmicrosomatic, were thought previously to show complex olfactory communication only in prosimians and in some New World monkeys. Now, it is known that even higher primates use pheromones to recognize conspecific individuals and to mark territory (Michael and Keverne 1968; Michael et al. 1971; Keverne 1983). Furthermore, protein–pheromone complexes present in the secretions released by scent marking of some nonhuman primates have been shown to activate vomeronasal receptors, particularly for sexually related behaviors and intraspecific identification of individuals (Smith et al. 2001; Hayes et al. 2004; Palagi et al. 2004; Vaglio et al. 2004).

Formerly, it was widely held that the human vomeronasal organ was vestigial, and even the existence of pheromonal communication in humans was contested. Support for a role of pheromones in human behavior came from observational studies, e.g., synchronization of the menstrual cycle (Mc Clintock 1971), but this role was often denied because there seemed to be an insufficient neuroanatomic basis for such complex behavior (Pelucchi 2006).

Recently, a new class of olfactory receptors (trace amino-associated receptors) was discovered in the olfactory epithelium of mice (Liberles and Buck 2006). Genes similar to those responsible for the control of these receptors in mice have been identified in humans and fish, which suggest evolutionary conservation and lend support to the hypothesis that the human pheromonal response is mediated by receptors located in the main olfactory system (Pelucchi 2006).

Putative human pheromones are steroids present in the secretions of exocrine glands (Taylor 1994; Stern and Mcclintock 1998; Grosser et al. 2000; Pause 2004; Grammer et al. 2005; Hauser et al. 2005). Estrogen derivatives are present in females (the so-called copulins—mixtures of aliphatic acids such as acetic, propionic, butyric, isovaleric, and isocaproic acid with estratetraenol), and androgen derivatives are present in males (androstenol, androstenone, and androstadienone). Studies concerning the most volatile compounds of human sweat (Zeng et al. 1991, 1996a, b; Bernier et al. 2000; Curran et al. 2005) have shown that the characteristic odor produced by the para-axillary region is due to the presence of volatile C6–C11 acids; the most abundant is *E*-3-methyl-2-hexenoic acid.

Human pheromones also may play a role in offspring identification. Odor cues from newborns are salient to their mothers (Kaitz et al. 1987). Mothers can distinguish the odor of their own newborn baby from that of other newborns (Schaal et al. 1980; Kaitz et al. 1987; Chiarelli 2001). Experiments also have demonstrated that adults can recognize gender and individuality of nonrelated children (Ligabue Stricker 1991; Chiarelli 2001). Thus, body odors can provide humans with information about the individual identity of their offspring (Doty 1981; Porter and Moore 1981; Porter et al. 1983, 1985; Russell et al. 1983; Curran et al. 2005; Olsson et al. 2006).

Children usually prefer parts of clothes that have been in contact with the axilla and worn by their own mothers to clothes worn by other mothers (Schaal et al. 1980). Thus, pheromones seem to have a fundamental role in the mechanism of mother–child identification (Porter et al. 1983; Porter and Winberg 1999). Breast-fed versus bottle-fed infants show different reactions to maternal odors. Breast-fed infants are exposed to salient maternal odors and rapidly become familiarized with their mother's unique olfactory signature (Cernoch and Porter 1985). Apparently, orientation to lactating-breast odors is an inborn adaptive response of a newborn (Porter et al. 1991).

Without doubt, naturally occurring odors play a role in mediating infant behavior. Even fetal olfactory learning seems to occur (Varendi et al. 1996), and breast odors from the mother exert a pheromone-like effect at the newborn's first attempt to locate the nipple. Newborns generally are responsive to breast odors produced by lactating women (Porter and Winberg 1999). Olfactory recognition may be implicated in the early stages of the mother–infant attachment process, when newborns learn to recognize the own mother's unique odor signature: This process is facilitated possibly by high norepinephrine release and the arousal of the locus coeruleus at birth (Winberg and Porter 1998). Human infants are responsive to maternal odors that begin shortly after birth. They show an attraction to amniotic fluid (AF) odor that may reflect fetal exposure to that substance (i.e., prenatal olfactory learning; Varendi et al. 1996). Moreover, human AF seems to carry individualized odor properties, which are hypothesized to initiate parent–infant interactions (Schaal and Marlier 1998).

On the basis of these literature findings, we hypothesized that women probably develop a volatile profile through pregnancy and childbirth that enables identification of the mother by the newborn. The aim of the present research was to understand how the volatile pattern of pregnant women changes during pregnancy and, consequently, provide the possible chemical basis for mother–infant olfactory recognition.

Methods and Materials

Sampling Procedures Sweat samples were collected from a group (N=20) of 30- to 40-year-old pregnant women (mean

age=36.8 year). Sweat patches (PharmChem Inc., Fort Worth, TX, USA) were applied to para-axillary and nipple–areola regions for at least 24 h. The patch is basically a large band aid with an untreated cellulose pad that is sterile and devoid of chemical additives. Blank patches were prepared under the same experimental conditions and used as controls. Isopropanol was used to clean the skin prior to application of the patch. The patches can be worn for up to 7 days, with a minimum of 24 h, whereas patch "overlays," included in the kit, can be placed over the original to provide even more adhesion.

Samples were collected between July 2006 and October 2007 from three periods during pregnancy and after childbirth to permit an evaluation of the volatile pattern during and after the gestational period: (1) the second month of gestation (7–10th week of gestation), (2) the last month of gestation (at about the 35th week of gestation), and (3) after childbirth (at least 6 months after birth). These particular three-time periods were selected on the basis of pragmatic criteria: (1) the earliest possible time period during pregnancy (based on first awareness of pregnancy), (2) the latest possible time period during pregnancy (based on avoiding the risk of premature births), and (3) a distant time period after pregnancy (based on the need for the subject women to begin their regular rhythm of life and, above all, their menstrual cycles).

All participants in the study signed an informed consent form for this totally noninvasive investigation. Italian protocols for human subjects were followed. The participants also responded to a short questionnaire. On the first day, a brief personal obstetrician anamnesis (personal history) was developed, and on the second, questions were asked regarding potential food and/or emotional anomalies (e.g., stressful occurrences, sexual activity) encountered during the period of sweat patch application that could potentially modify the pheromonal profile. Sample collection was carried out from the para-axillary and nipple–areola regions with the following protocol: (1) Banned fragrances and deodorants were not used in the previous 24 h; (2) unusual food and/or stresses and emotional behaviors were not present in subjects; (3) sweat patches were applied, one to the para-axillary and one to the nipple–areola region; (4) sweat patches were removed after at least 24 h and inserted into 20-ml solid phase microextraction (SPME) vials, which were hermetically closed by teflon-faced rubber septa and aluminium seals with a fit hand crimper; and (5) vials with the sweat patches were maintained at −20°C and were transported to the laboratory for analysis.

Extraction, Characterization, and Quantification of Volatile Compounds A SPME syringe needle, coated with 30 μm of polydimethylsiloxane, was introduced through the vial septum (Supelco, Bellefonte, PA, USA). The vial was held at 60°C, and the fiber was exposed to the headspace above the sample for 20 min. Adsorbed volatile analytes were analyzed by gas chromatography–mass spectrometry (GC-MS) by using a DSQ mass spectrometer (Thermo Electron Corporation, Waltham, MA, USA) operated in electron impact (EI; 70 eV) mode and directly coupled to a Focus gas chromatograph (Thermo Electron Corporation, Waltham, MA, USA). The GC was equipped with a fused silica HP 5-MS capillary column (30 m×0.25 mm crossbonded 5% phenyl–95% dimethylpolysiloxane, film thickness 0.50 μm, Agilent Technologies, Santa Clara, CA, USA). The injector and transfer line temperatures were maintained at 220°C and 250°C, respectively. Injections were made in splitless mode with a constant flow of helium carrier gas of 1.1 ml/min. The oven temperature program, started at 40°C, was held for 3 min and then raised by 10°C/min to 100°C and in a second step by 5°C/min to the final temperature of 250°C. The eluted compounds were tentatively identified by comparing the experimental spectra with those of the NIST mass spectral library, Version 2.0 (Thermo Electron Corporation, Waltham, MA, USA). Authentic standards for confirmation of compound identities were purchased from Sigma Aldrich, Milan, Italy. The relative amounts of the compounds of interest were determined by integrating the areas of the corresponding peaks in the total ion current (TIC) profile. Percentages were calculated with respect to the most abundant compound.

Statistical Analyses The peaks in the TIC profile were taken as measures of the relative amounts of the volatile compounds of interest. Each compound was considered as a variable and, for further analysis, we assigned each compound a categorical value of 0 or 1: 1 indicated the presence of compound and 0 its absence. Questionnaires provided information about lactation, menstrual cycle, and the contingent occurrence of food and/or emotional anomalies.

The goal of the statistical analysis was to determine whether the presence/absence of the five volatile compounds was influenced by body area (A = para-axillary region, B = nipple–areola region), sampling time (1 = beginning of gestation, 2 = end of gestation, or 3 = after childbirth), and other variables collected on the questionnaire as listed above. To study these possible associations, we used a multiple logistic regression model, a particular type of generalized linear model for binary data that is used to study the relationship among dependent variables (also known as response and independent binary variables; statistical package "R", Version 2.7.2, Free Software). The dependent variable was the presence/absence of each volatile compound (1-dodecanol, 1-1′-oxybis octane, isocurcumenol, α-hexyl-

cinnamic aldehyde, or isopropyl myristate, see below), whereas the independent variables were the body area, the sampling time, the contingent occurrence of food and/or emotional anomalies, and the possible presence of lactation and/or menstrual cycle.

A reweighted least squares method (statistical package "R", Version 2.7.2, Free Software) was used to fit the data in the regression model. Statistical significance of the associations was determined by analysis of variance (α= 0.1 for significance; statistical package "R", Version 2.7.2, Free Software).

Results

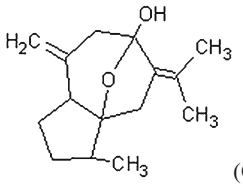
Identification of Volatile Compounds During pregnancy, all women developed a distinctive volatile pattern represented by five compounds common to the para-axillary and nipple–areola regions (see below). The five compounds (R_t) are (**A**) 1-dodecanol (17.83 min), (**B**) 1-1′-oxybis octane (21.60 min), (**C**) isocurcumenol (21.79 min), (**D**) α-hexyl-cinnamic aldehyde (23.27 min), and (**E**) isopropyl myristate (24.54 min). Identification of all commercially available compounds (**A**, **B**, **D**, **E**) was confirmed by comparison of the retention times and mass spectra with those of authentic standards. Compound **C** was identified as isocurcumenol by comparison with the EI mass spectrum of isocurcumenol reported in the literature (Yang et al. 2005), where the mass spectrum of the isomer curcumenol also is reported: The fragmentation of the two isomers is completely different.

$CH_3(CH_2)_{11}OH$ (**A**)

$[CH_3(CH_2)_7]_2O$ (**B**)

$C_6H_5CH{=}C[(CH_2)_5CH_3]CHO$ (**D**)

$CH_3(CH_2)_{12}COOCH(CH_3)_2$ (**E**)



(**C**)

The five compounds, which were absent in the analysis of blank patches, were the only recurrent volatile compounds within the three sample periods. They were not detected in an equivalent control sample of nonpregnant and nonlactating females (Fig. 1). Thus, the volatile composition changes during pregnancy.

Effect of Body Area, Time, Lactation, Menstrual Cycle, Food, and/or Emotional Anomalies The effects of various independent variables from the multiple logistic regression

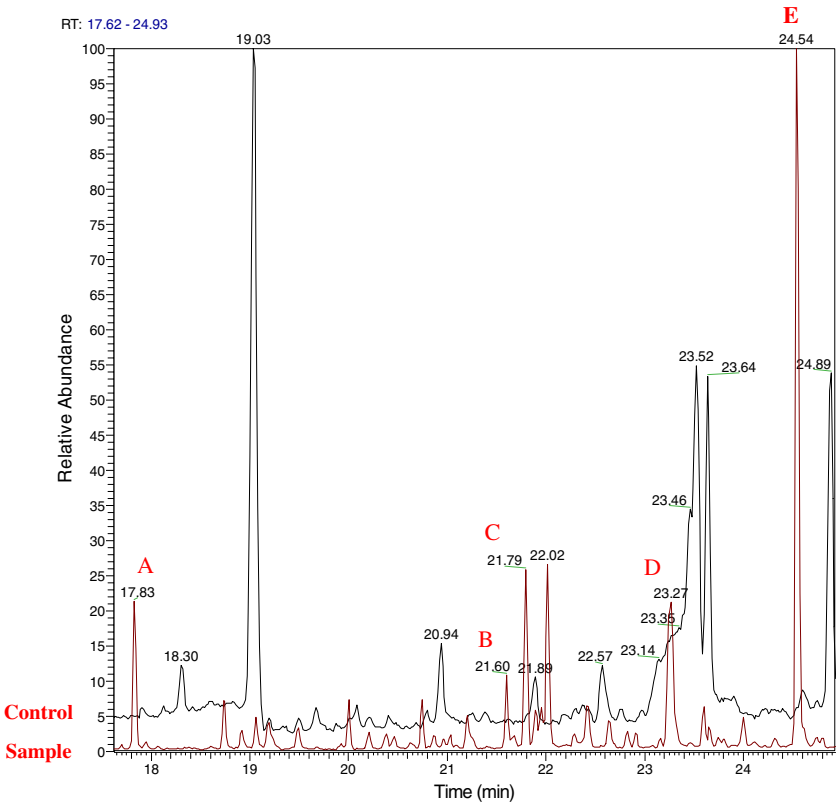


Fig. 1 TIC profile of a nipple–areola sample during pregnancy (*red*/*bottom*) compared to a similar sample from a nonpregnant and nonlactating control (*black*/*top*). The range corresponds to the elution of the five frequently occurring compounds (1-dodecanol, R_t=17.83 min, *A*; 1-1′-oxybis octane, R_t= 21.60 min, *B*; isocurcumenol, R_t=21.79 min, *C*; α-hexyl-cinnamic aldehyde, R_t=23.27 min, *D*; isopropyl myristate, R_t= 24.54 min, *E*). It focuses on the period between R_t=17.60 and 25.00 min that has been normalized. The unlabeled peaks reflect compounds not common to all samples

on the dependent variables (presence/absence of 1-dodecanol, 1-1′-oxybis octane, isocurcumenol, α-hexyl-cinnamic aldehyde, or isopropyl myristate) are as follows:

1. The occurrence of lactation during the third sampling period (after childbirth) did not affect the presence/absence of any volatile compound (1-dodecanol, $P=1$; 1-1′-oxybis octane, $P=0.400$; isocurcumenol, $P=0.209$; α-hexyl-cinnamic aldehyde, $P=0.270$; or isopropyl myristate, $P=1$), nor did the return of the menstrual cycle (1-dodecanol, $P=0.209$; 1-1′-oxybis octane, $P=0.400$; isocurcumenol, $P=0.209$; α-hexyl-cinnamic aldehyde, $P=0.270$; or isopropyl myristate, $P=1$).
2. No independent variable (body area, sampling time, food, and/or emotional anomalies) affected the presence of α-hexyl-cinnamic aldehyde (model with food anomalies as independent variable—body area, $P=0.142$; second sample period, $P=0.998$; third sample period, $P=0.788$; and food, $P=0.469$; model with emotional anomalies as independent variable—body area, $P=1$; second sample period, $P=0.998$; third sample period, $P=0.584$; and emotions, $P=0.436$).
3. No independent variable (body area, sampling time, food, and/or emotional anomalies) affected the presence of isopropyl myristate (model with food anomalies as independent variable—body area, $P=1$; second sample period, $P=1$; third sample period, $P=1$; and food, $P=1$; model with emotional anomalies as independent variable—body area, $P=1$; second sample period, $P=1$; third sample period, $P=1$; and emotions, $P=1$).
4. The combination of body area with food anomalies ($P=0.066$) and emotional anomalies ($P=0.046$) affected the presence of isocurcumenol.
5. The combination of sampling time with food anomalies ($P=0.051$) and emotional anomalies ($P=0.073$) affected the presence of 1-1′-oxybis octane.
6. The combination of sampling time and emotional anomalies ($P=0.053$) affected the presence of 1-dodecanol.

Additionally, a chi-square test supports these conclusions (data not shown). Since the control values were equal to 0, they were not included in the statistical analysis.

Qualitative Analyses The patterns of the relative occurrence of the five volatile compounds in the samples varied with the body regions and the time of sampling (Fig. 2). In early gestation, nearly all samples from the nipple–areola region contained all five volatile compounds. Fewer samples from the para-axillary region had the compounds during early gestation. In late gestation, the five components were detected in nearly all samples from both body areas, whereas after childbirth, the fraction of samples that contained the compounds declined dramatically in both body areas. After childbirth, 1-1′-oxybis octane was not detected in any of the nipple–areola region samples and in only 20% of the para-axillary samples. Only isopropyl myristate was present at a high frequency in the samples after childbirth.

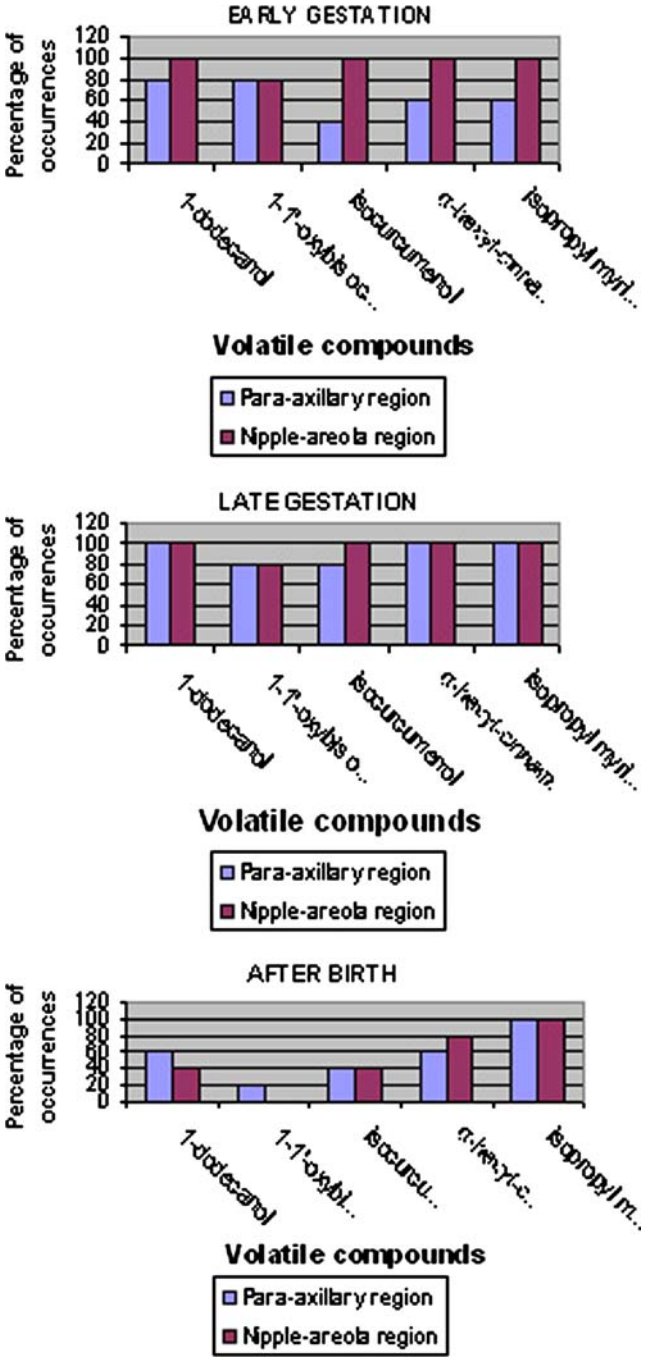


Fig. 2 Comparison of relative frequency of occurrence of volatile compounds in samples from the para-axillary and nipple–areola regions from women ($N=20$) during three sampling periods

Relative Quantitative Analyses The amounts of the five compounds relative to the most abundant compound in each

sample also varied with sampling period in the two different body areas. The para-axillary region (Fig. 3) was characterized by a pattern with generally high levels of 1-dodecanol, 1-1′-oxybis octane, isocurcumenol, and α-hexyl-cinnamic aldehyde during pregnancy and a subsequent decline in abundance to nearly complete disappearance of most of these compounds after childbirth. The relative amount of isopropyl myristate in the para-axillary samples was high during pregnancy and increased after childbirth. The nipple–areola region (Fig. 4) was characterized by a similar pattern but with greater relative amounts of isocurcumenol during late gestation and lower relative amounts of isopropyl myristate during pregnancy.

Discussion

Our data showed that during pregnancy, all women developed a distinctive olfactory pattern that involved at least five volatile compounds. This pattern was absent in

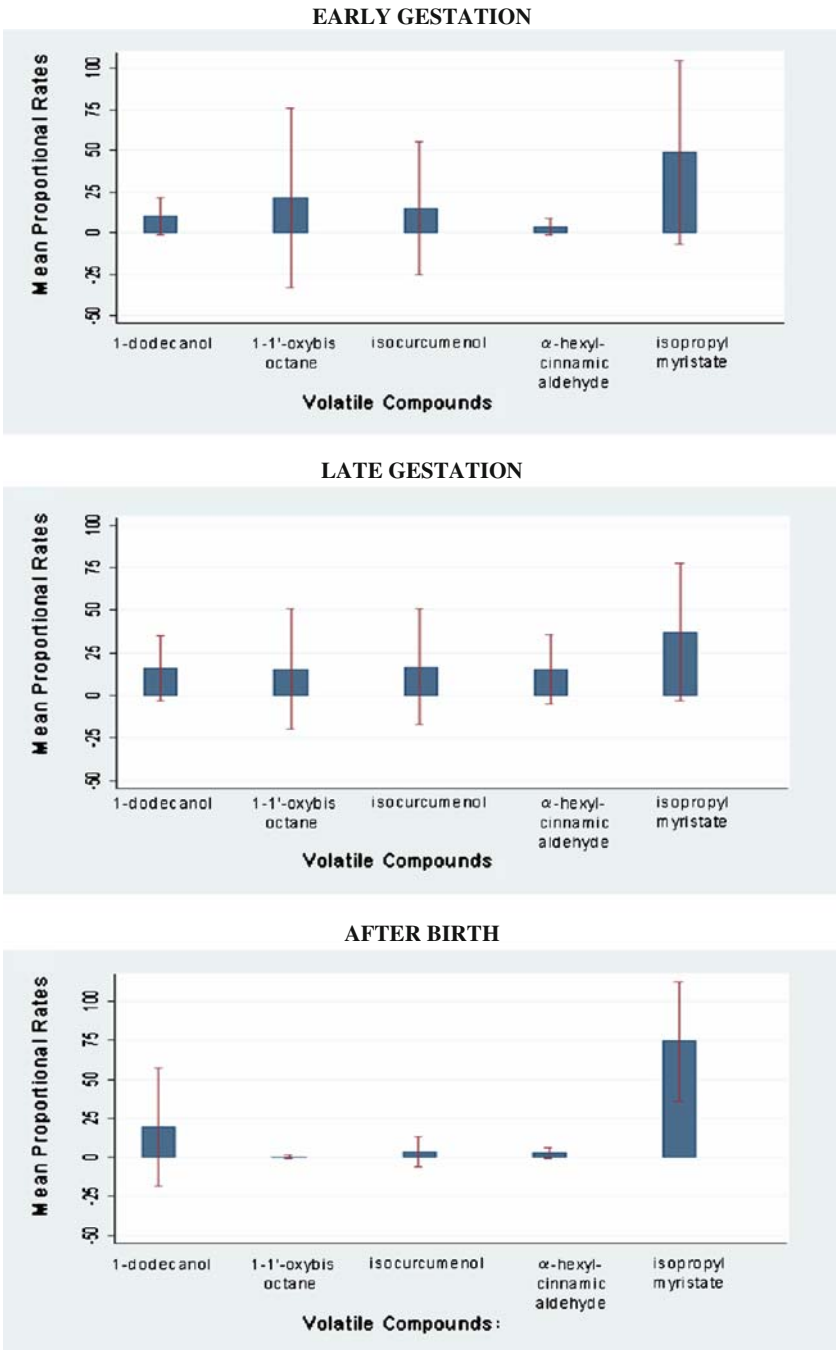


Fig. 3 Relative amounts (mean ± 2 standard error, N=20) of five volatile compounds in samples from the para-axillary region during three sampling periods

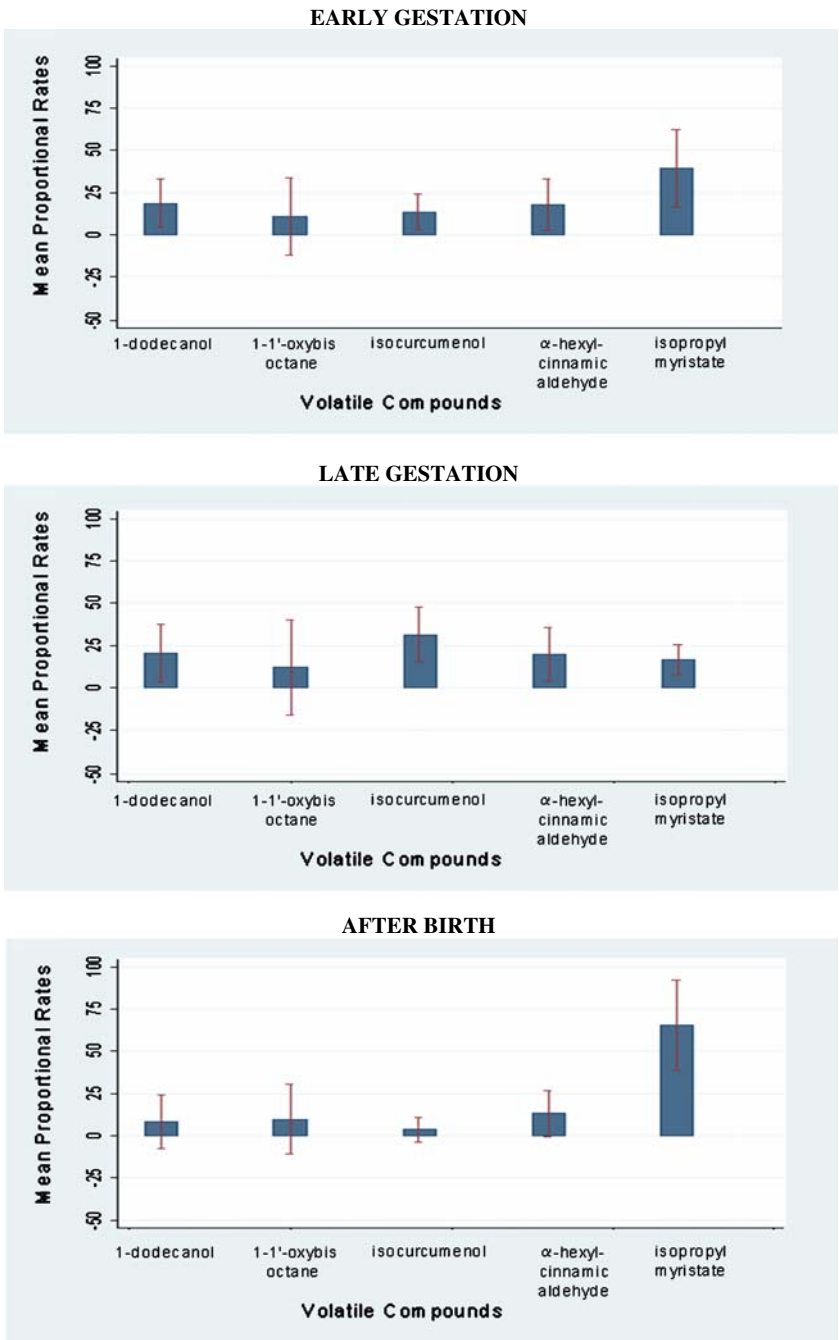


Fig. 4 Relative amounts (mean ± 2 standard error, N=20) of five volatile compounds in samples from the nipple–areola region during three sampling periods

samples from nonpregnant and nonlactating control subjects but was present in samples taken during pregnancy as well as after childbirth. The compounds were present in samples from both the para-axillary and the nipple–areola regions. The precise time periods selected for sample collection were chosen on the basis of pragmatic criteria. The collection times that produced the above mentioned results were not fixed points but a series of "windows" suitable for collection.

From a qualitative point of view (Fig. 2), we can infer that α-hexyl-cinnamic aldehyde and isopropyl myristate remain constant like a matrix in the pattern, isocurcumenol functions as a distinctive signal between para-axillary and nipple–areola regions, and 1-dodecanol and 1-1′-oxybis octane behave as indicators for different periods of the pregnancy. Surprisingly, after childbirth, this pattern did not appear to be influenced by factors such as lactation or the menstrual cycle but was modified by food and emotional anomalies. In particular, 1-1′-oxybis octane was affected by food anomalies; 1-1′-oxybis octane and 1-dodecanol were affected by emotional anomalies (data not shown). From the relative quantitative results, we infer that isopropyl

myristate represents a matrix of the pattern in both body regions and during all three sample periods, whereas 1-dodecanol, 1-1′-oxybis octane, isocurcumenol, and α-hexyl-cinnamic aldehyde behave as indicators for different phases of pregnancy, contemporaneously working as distinctive signals between the para-axillary and nipple–areola regions.

The presence of these volatile compounds and the related pheromonal role for some of them in other species may have implications from the point of view of evolution and phylogeny. First, three of the compounds identified in this study (oxybis octane, isocurcumenol, and α-hexyl-cinnamic aldehyde) have not been found previously in any other animal species. Oxybis octane for instance has apparently never been found to have a significant biological role, and isocurcumenol is rather widespread but as a volatile compound produced by some plants (Ha et al. 2002; Yang et al. 2005; Zhou et al. 2007). α-Hexyl-cinnamic aldehyde has frequently been utilized as a fragrance in both cosmetic and detergent products (Rastogi et al. 1996; Kanei et al. 1999). Dodecanol and isopropyl myristate have been suggested previously to have a pheromonal role in both humans and other animal species. For example, 1-dodecanol has been identified as a volatile compound from the plant *Houttuynia cordata* Thunb. (Liang et al. 2005), as precursor of the main sex pheromone of the female of the crab, *Erimacrus isenbeckii* (Masuda et al. 2002), as a pheromone in the anogenital marking of males and females of *Lemur catta* (Hayes et al. 2004), and as a volatile compound of human sweat (Meijerink et al. 2000, 2001). Isopropyl myristate is one of the main volatile compounds produced by the human axilla, and it is also often utilized as a component of deodorants and other cosmetic products (Labows et al. 1979). This compound is a volatile component of the head, thorax, and alimentary canal of the ant *Iridomyrmex humilis* (Cavill and Houghton 1974) and as a sex pheromone that (together with heneicosane, docosane, nonacosane, and octadecanes) stimulates oviposition in the mosquito, *Aedes aegypti* (Corkum and Belanger 2007).

Based on our results, we hypothesize that the distinctive chemical pattern of the para-axillary area could be useful for newborns to recognize their own mothers and distinguish them from other individuals. The pattern of volatiles from the nipple–areola region may function as an aid to finding sustenance. The results also demonstrate the effectiveness of the methodology. By using this collection and analysis method, we investigated the volatile compounds in a systematic way. Previously, such phenomena often have been investigated with inadequate methodologies, and as a consequence, the role of volatile compounds likely has been underestimated. The behavioral mechanisms related to these potential chemical signals bear future investigation in humans.

There are practical implications of the present research. The study of the mechanism of mother–child identification is important for the acquisition of new knowledge concerning the emission of signal molecules essential for mother–child identification and also for setting the proper conditions for establishing solid mother–child bonding. An understanding of the mechanisms of newborn recognition of mothers also could have practical health implications. Moreover, the research provides a basis for testing hypotheses about the role of pheromones in the evolution and phylogeny of humans. It seems apparent that pheromones influence a wide range of reproductive behavior, a central theme to the survival of any species, a subject long underestimated for its impact on human behavior and evolution.

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Is (−)-Catechin a Novel Weapon of Spotted Knapweed (*Centaurea stoebe*)?

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Abstract The novel weapons hypothesis states that some invasive weed species owe part of their success as invaders to allelopathy mediated by allelochemicals that are new to the native species. Presumably, no resistance has evolved among the native species to this new allelochemical (i.e., the novel weapon). In their native habitat, however, the plants that co-evolved with these invasive species have theoretically evolved defenses that obviate the allelochemical advantage. Previous studies have claimed that catechin is such a novel weapon of spotted knapweed (*Centaurea stoebe* = *C. maculosa*), an invasive species in the non-native habitat of North America. These studies indicated that (−)-catechin is more phytotoxic than (+)-catechin. Other studies have not found sufficient catechin in field soils to support this theory. We report that (−)-catechin and (+)-catechin are essentially equal, but poorly phytotoxic to a variety of plant species in bioassays without soil. In a dose/response experiment with Montana soils, we found the lowest dose for a growth reduction of two native Montana grasses (*Koeleria macrantha* and *Festuca idahoensis*) by a racemic mixture of (±)-catechin that ranged from about 25 to 50 mM, concentrations, orders of magnitude higher than expected in nature. Autoclaving the soil before adding the catechin did not affect the activity of catechin. We found (−)-catechin to be a potent antioxidant, in contrast to a previous claim that it acts as an allelochemical by causing oxidative stress. Our findings suggest that catechin is not a novel weapon of spotted knapweed and that other allelochemical(s) or alternative mechanisms must be found to explain the success of this species as an invader in North America.

Keywords Allelochemical · Allelopathy · Catechin · *Centaurea maculosa* · *Centaurea stoebe* · Invasive species · Phytotoxin · Soil · Spotted knapweed

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Introduction

Some invasive plant species are apparently allelopathic and may owe part of their success in new habitats to this trait (Weston and Duke 2003). Claims have been made that the success of *Centaurea stoebe* (= *C. maculosa*; spotted knapweed) in North America results from allelopathy via the release of (−)-catechin [(2*S*,3*R*)-(−)-catechin] (Bais et al. 2002, 2003; Weir et al. 2003; Thelen et al. 2005; Callaway and Ridenour 2004; Inderjit et al. 2008). This compound was reported to be exuded from the roots of spotted knapweed, and it was identified as the putative allelochemical of this invasive species (Bais et al. 2002). The (+)-catechin enantiomer [(2*R*,3*S*)-(+)-catechin] is also

produced by the plant, but was initially reported to be nonphytotoxic (Bais et al. 2002) and later 1.5 to two times less active than (−)-catechin (Veluri et al. 2004, erratum).

Catechin is a commonly found flavonoid present in fruits, wine, vegetables, and cocoa products, as well as in many nonfood plants. Grape seeds contain a significant amount of (+)-catechin and (−)-epicatechin. Most foods contain the (+)-enantiomer of catechin, but chocolate mainly contains (−)-catechin together with (−)-epicatechin. (+)-Catechin is the most commonly found enantiomer in nature. Both enantiomers possess antioxidant properties. The two enantiomers have identical nuclear magnetic resonance (NMR) spectra and high-performance liquid chromatography (HPLC) retention times and identical R_f values in thin layer chromatography (TLC). The only difference is in optical rotation and the CD spectra, where these two compounds rotate plane polarized light equally in opposite directions. The CD spectra of these enantiomers are antipodal. The mirror image differences in molecular shape can result in differences in biological activity (e.g., Donovan et al. 2006).

Multiple studies have examined various aspects of the role of (–)-catechin as an allelochemical (Bais et al. 2003; Weir et al. 2003, 2005; Veluri et al. 2004; Callaway et al. 2005; Perry et al. 2005; Thelen et al. 2005). This work, in part, laid the groundwork for the novel weapons hypothesis, which states that the success of some exotic invasive plant species may be due to the production of allelochemicals that native species have never encountered and, thus, to which they have not evolved defenses (Callaway and Ridenour 2004).

Blair et al. (2005, 2006, 2009) questioned the findings of (−)-catechin as the allelochemical responsible for the successful invasion of spotted knapweed. They found that the methods for catechin extraction described in previous papers (Bais et al. 2002, 2003; Weir et al. 2003) could not extract catechin from liquid media and produced low recovery efficiencies from soil. With methods that were much more efficient for catechin extraction, they quantified levels of production up to two orders of magnitude less with plants grown in media than stated in earlier publications. A grass species (*Festuca idahoensis*) native to some of the spotted knapweed-infested areas of North America was only slightly affected by racemic catechin concentrations 20-fold higher than those previously reported to cause 100% mortality (Blair et al. 2005). Lastly, no measurable concentrations of catechin could be detected in soil samples from two spotted knapweed sites in Montana, USA. The last issue was countered with the argument (Inderjit et al. 2006) that catechin can be found only at certain times in these soils and that Blair et al. (2005) had sampled at the wrong time of the year (October, 2004). Blair et al. (2006) sampled three field sites through the summer and fall of 2005 and detected catechin at only one time point at two of the field sites at levels, three orders of magnitude lower than what they had previously reported to reduce growth of *F. idahoensis*. Later, Perry et al. (2007) found little or no catechin in 402 soil samples from 11 *C. stoebe* sites, except on one sampling date, when all of the samples from one site had levels of catechin of 0.65±0.45 (SD) mg g^{-1}. In this paper, the authors were less firm than they were previously in their hypothesis that catechin plays a role in the allelopathy of spotted knapweed.

Although many natural compounds can be shown to be phytotoxic in the laboratory or greenhouse, to prove allelopathy, it is ultimately necessary to demonstrate the presence of the compound under natural field conditions at concentrations high enough to have an impact on neighboring plants (Cheng 1995; Romeo 2000). In solution, catechins can be phytotoxic to plants, including algae (Buta and Lusby 1986; D'Abrosca et al. 2006), but there is inadequate evidence of its activity in soil. In this paper, we present evidence that is consistent with the findings of Blair et al. (2005, 2006, 2009) and others (Tharayil et al. 2008). We report that both (+)- and (−)-catechin are weak phytotoxins on a variety of plant species in liquid or agar cultures and that they are essentially inactive as phytotoxins in soil.

Methods and Materials

Plant Material Lettuce seeds (*Lactuca sativa* L. cv. Iceberg A) were obtained from Burpee Seed Company and creeping bentgrass seeds (*Agrostis stolonifera* var. Penncross) from Turf-Seed (Hubbard, OR, USA). Duckweed (*Lemna paucicostata* Hegelm. 6746) was from a stock maintained in our laboratory since it was brought to us from Japan (Matsumoto and Duke 1990). These species were selected because we have used them to evaluate thousands of synthetic and natural compounds for phytotoxicity (e.g., Dayan et al. 2000, Michel et al. 2004).

Arabidopsis thaliana (accession Columbia-0/Redei-L206440) seeds were obtained from Lehle Seeds (Round Rock, TX, USA). *Arabidopsis* was used because the original papers that claimed (−)-catechin to be a novel weapon used it as one of their test species (Bais et al. 2002, 2003).

Seeds of junegrass (*Koeleria macrantha*) and Idaho fescue (*F. idahoensis*) were obtained from Western Native Seed (P. O. Box 188, Coaldale, CO, USA). These species were selected because they are native to the areas infested by spotted knapweed in North America, and they had been reported previously to be sensitive to catechin (Bais et al. 2003).

Chemicals The three catechins used [(+)-catechin hydrate, (−)-catechin, and (±)-catechin hydrate] were obtained from Sigma-Aldrich, for experiments in which small amounts were needed. The enantiomeric structures of (−)- and (+)-catechin in different lots of these chemicals were verified by circular dichroism spectroscopy (JASCO, Model J-715). All concentrations of catechins used in nonsoil bioassays were prepared by serial dilution from 10 mM stock solutions prepared in 5% (*v*/*v*) acetone. Epicatechin, catechinic acid, mannitol, and quercitin also were purchased from Sigma-Aldrich.

For soil studies, (−)–catechin was synthesized by adding 5 g of (−)-epicatechin to N_2 gas-saturated aqueous 5% NaOH (*w*/*v*) (75 ml) and stirring under N_2 for 10 min at 65°C. The mixture was cooled on ice, acidified to about pH 3 with about 60 ml of ice cold 2 N HCl, and extracted with ethyl acetate (3×200 ml). The organic layer was dried over anhydrous Na_2SO_4, and the viscous solution (ca 30 ml) was applied to a Sephadex LH-20 column (35 mm id×40 cm) equilibrated with ethanol/H_2O (85:15, *v*/*v*). The fractions were monitored by TLC and HPLC using (+)-catechin as the reference. The two enantiomers have the same retention time. (−)-Catechin-enriched fractions were pooled and concentrated to obtain 1.5 g of material. This was further purified with a C-18 gravity column (12 cm× 3.4 cm id) equilibrated with 10% MeOH and water. (−)-Catechin-enriched fractions were pooled, evaporated at 40°C to remove MeOH, freeze dried, and crystallized with methanol and CH_2Cl_2 to yield 420 mg of white needle-like crystals. The identity of the compound was confirmed as catechin by HPLC and NMR, and the absolute configuration was determined by comparison of CD spectra with those of (+)- and (−)-catechin.

Bioassays without Soil Lettuce seeds were surface disinfected with a 10% Chlorox® solution (6.15% *w*/*v* sodium hypochlorite) for 20 min, followed by 1 h of continual rinsing with sterile distilled water. Seeds were air-dried overnight in a Nuaire Biological Safety cabinet. Sterile techniques were used when handling.

Two milliliters of the appropriately diluted solutions of (−), (+), or (±)-catechin (initially dissolved in acetone to give a 5% acetone concentration in the final dilution) were added under sterile conditions to a 9-mm circle of sterile filter paper (Whatman no. 1) in a sterile, disposable Petri dish (Falcon no. 351005 Optilux Petri dish 100×20 mm). A solvent control set of dishes was prepared in the same manner using 5% acetone. In one experiment, the pH values of the test solutions were adjusted with either 0.1 M NaOH or 0.1 M HCl, depending on the pH desired. Fifteen lettuce seeds were added to the moistened paper. The dish was covered, sealed with a strip of parafilm, wrapped in aluminum foil to prevent light from entering, and then placed in a dark incubator (Percival Scientific, Model E-30LED3) at 25°C for a 7-day incubation period. Growth was in darkness in order to obtain maximal hypocotyl growth. Four replicates were prepared for each catechin concentration and the control. After 7 days, the dishes were removed, unwrapped from the foil, and placed under light in another incubator (Percival Scientific, Model CU-36L5) at 25°C for 1 day in order for chloroplast development to occur. After the 24-h light cycle, the dishes were removed from the incubator, and the root and hypocotyl lengths were determined. In all treatments, including the control, there were a few ungerminated and/or stunted seedlings, so only the ten most robust plants in each dish were measured. Averages from the measurements from the ten plants per dish were determined. The average of the four means and SE of the means from the four dishes were plotted. Bentgrass bioassays were conducted as previously described (Dayan et al. 2000).

Highly phytotoxic compounds, especially those that cause photooxidative damage, eventually cause loss of chlorophyll. Chlorophyll analysis was done by extracting at least 10 mg of lettuce cotyledon or bentgrass leaf tissue with 2 ml of dimethyl sulfoxide (DMSO) for 1 h at 60°C to 62°C according to the method of Hiscox and Israelstam (1980). There was no further diluting necessary prior to spectrophotometric analysis (Shimadzu UV-3101 PC UV-VIS-NIR). The chlorophyll–DMSO extracts were cooled to room temperature and absorbance at 645 and 663 nm measured with DMSO as the blank. Chlorophyll concentration was determined by the equation of Arnon (1949). The average of the chlorophyll content in lettuce cotyledons or bentgrass leaves was determined.

Duckweed plants were grown and treated with catechins as previously described for herbicides (Michel et al. 2004). Growth was determined nondestructively by image analysis of frond area as described in detail by Michel et al. (2004). Each treatment was conducted in quadruplicate, with SEs of means generated from the data.

A. thaliana plants were treated with catechin in a manner similar to that of Bais et al. (2003). Seeds were sterilized by the method of Baerson et al. (2005) and then placed in filter paper-lined, 6-cm Petri dishes in 3 ml of half strength MS medium (Murashige and Skoog 1962) under a 16:8-h L:D photoperiod at 21°C. The plates were sealed with 3 M surgical tape. Plants were grown for 10 days under these conditions, and then the most robust plants were transferred to 24-well plates (three seedlings per well) containing half strength MS medium with different amounts of (+), (−), or (±)-catechin (initially dissolved in acetone to give a 5% final acetone concentration) or control wells. There were 12 wells of three plants each for each treatment. After 4 days, root lengths were determined, and then all plants were replaced in 24-well plates with half strength MS medium without catechins for 4 days.

In another experiment, square Petri dishes (9×9×1.5 cm) were used for *A. thaliana* bioassays as before (Baerson et al. 2005) with the following modifications. Seeds were germinated and seedlings grown on sterile, semisolid Gamborg's B5 (Gamborg et al. 1968) at a pH of 5.7 with minimal organics (Sigma-Aldrich) supplemented with 10 g/l sucrose. The media of some of the plates was amended with (+)-catechin, (+/−)-catechin, or (−)-catechin. Acetone was used as a solvent where each plate contained 1.0% (*v*/*v*) acetone. The controls contained the same amount of acetone. Plants were grown in a controlled environment chamber (Percival) under a photoperiod of 16 h light illuminated at 100 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) and 8 h dark at 22°C for 14 d. Photographs were taken at day 14.

Bioassays in Soil Metromix 350 potting soil (Hummert International, Earth City, MO, USA) was used in some studies. This mixture contains horticultural grade vermiculite, peat moss, processed bark ash, nutrients, dolomite limestone, and a wetting agent.

In other studies, soil from near the towns of Nelson Gulch and Jens, Montana collected in October, 2005 was used. GPS coordinates for the two sites are: Nelson Gulch 46°34.373′ N 112°8.820′ W 4,273 m and Jens Exit 46° 36.359′ N 113°00.832′ W 4,136 m. Further details of the collection sites are provided in Blair et al. (2006). These soils were shipped to Oxford, MS, USA, where they were stored at room temperature until use in bioassays. The storage time varied from days to months, depending on when the experiment was done. The Montana soils were screened to remove rocks and larger organic fragments with a sieve used for sifting flour and then finally screened with a USA standard testing sieve (Tyler 35 mesh equivalent, 425 μm). Ten grams of air-dried soil was placed in 6-cm Petri dishes with different amounts of (±)-catechin or (−)-catechin. Catechins were incorporated by sifting the soils several times with the appropriate amount of the dry chemical. In some studies, the soils were autoclaved (three cycles of 30 min at 121°C, followed by 30 min cooling) before use to eliminate potential effects of soil microflora on catechin. Seeds of junegrass or Idaho fescue pretreated with 0.2% (*w*/*v*) KNO_3 to induce uniform germination were planted (25 seeds/dish) in soils from Nelson or Jens soil to which 5 ml of distilled water were added. The dishes were then placed in growth chambers at 24°C with 14-h photoperiods and 70 μmol m^{-2} s^{-1} PAR. The five most robust plants from each dish were selected, and plant height, fresh weight, and, in some cases, dry weight were determined 10 to 13 days after planting.

Determination of Free Radical Quenching of Catechins The method of Cespedes et al. (2001) was used to detect ROS quenching of natural compounds. Silica gel TLC plates (GF_{254} 250 μm, Analtech) were spotted with approximately 2.5 μl (2 mg/ml in methanol) of (+)-catechin, (−)-epicatechin, (−)-catechin, the antioxidant quercetin (Hollman et al. 1997; Zhang et al. 2008), and the nonquenching compound mannitol. Plates were developed in toluene/acetone/methanol (6:3:1) with two drops of acetic acid per 10 ml. After eluting, TLC plates were dried and sprayed with a 0.2% 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution (*w*/*v*) in methanol. Plates also were exposed to iodine vapor. The plates were photographed 30 min after spraying. Antioxidant activity was evidenced by appearance of yellow spots against a purple background.

Analysis of Data Experiments were repeated in time and space. Data were analyzed with the SAS Software release 9.1 (SAS Institute, Cary, NC, USA). Analysis of variance was performed for each compound concentration, and means were tested with Duncan multiple range test. SEs are also provided to show the variation associated with particular means. Data from dose–response experiments were analyzed with the add-on package for dose–response curves (drc; Ritz and Streibig 2005), for R version 2.2.1 (R Development Core Team 2005) using a four-parameter logistic function. Means and SEs were obtained using the raw data, and I_{50} values were one of the parameters in the regression curves. The regression curves were imported into SigmaPlot version 10 (Systat Software, San Jose, CA, USA).

Results

Soil-free bioassays When *A. thaliana* was bioassayed in a manner similar to that of Bais et el. (2002, 2003), (+)-, (−)-, and (±)-catechin significantly inhibited root growth at 0.33 and 1 mM, with (−)-catechin being slightly more active than (+)-catechin (Fig. 1). As described by Bais et al. (2003), roots darkened after exposure to catechins (Fig. 2). When plants were transferred to a catechin-free medium after 4 days of catechin exposure, root growth resumed (Fig. 2). The new root growth was not dark, and the darkening in the old roots was less intense. Healthy root hairs were seen in all treatments (Fig. 3) after removal from catechin.

In agar, there was little or no growth reduction of *A. thaliana* roots or shoots when grown continuously on 0.17 or 0.35 mM (−) or (+) catechin, respectively (Fig. 4). There was marked root growth reduction at 0.7 mM, accompanied by browning of the roots. The effects were similar for both enantiomeric forms of catechin.

In solution, catechin can degrade to epicatechin and catechinic acid under some conditions (Kiatgrajai et al.

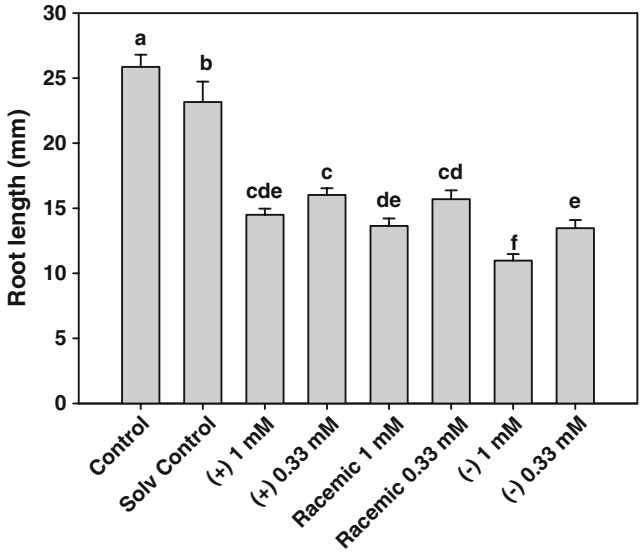

Fig. 1 Root lengths of *A. thaliana* plants grown for 10 days in MS medium and then transferred to 24-well plates for 4 days containing the indicated media. *Error bars* are ±1 SE of the mean. *Means with the same letters above them* are not statistically different at the α= 0.05 level, using Duncan's multiple range test

1982). We found neither of these compounds to be phytotoxic to lettuce, bentgrass, or duckweed at concentrations up to 1 mM (data not shown).

With bentgrass, there was a slight and similar reduction in chlorophyll with both (−)- and (+)-catechin at 3.3 mM (Fig. 5). Studies with duckweed found that both enantiomeric forms of catechin reduced growth at 3.3 mM and that the dose–response curves were similar for both enantiomers (Fig. 6). There was a slight reduction of lettuce hypocotyl growth by 333 μM (+)-catechin (Fig. 7a). Growth of lettuce roots was affected slightly, but significantly, by both enantiomers of catechin at 33 μM and more strongly by 333 μM (−)-catechin (Fig. 7b). Lettuce seedlings, germinated and grown for 8 days in 3.3 mM racemic catechin, were not appreciably stunted (Fig. 8), but browning of the hypocotyl and roots occurred. Chlorophyll levels of the cotyledons of lettuce were not affected at any concentration of any form of catechin tested (data not shown). The stability of catechin in water is affected by pH (Courbat et al. 1977; Hashida et al. 2003; Ho et al. 1995; Blair et al. 2005; Labrouche et al. 2005) with catechin becoming less stable above pH 5. We found

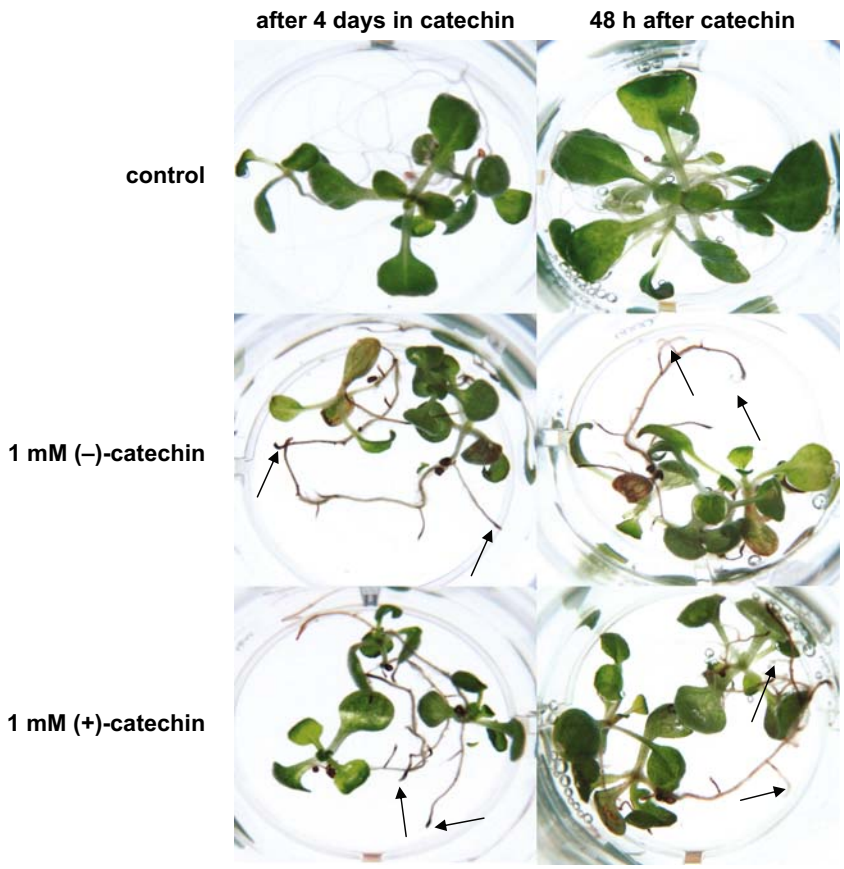

Fig. 2 *A. thaliana* plants grown for 10 days in MS medium and then transferred to 24-well plates for 4 days to the indicated media, after which all treatments were transferred to media without catechins. *Arrows in the first column of photographs* indicate darkened root tips. *Arrows in the second column of photographs* indicate white root tips after 2 days without catechin

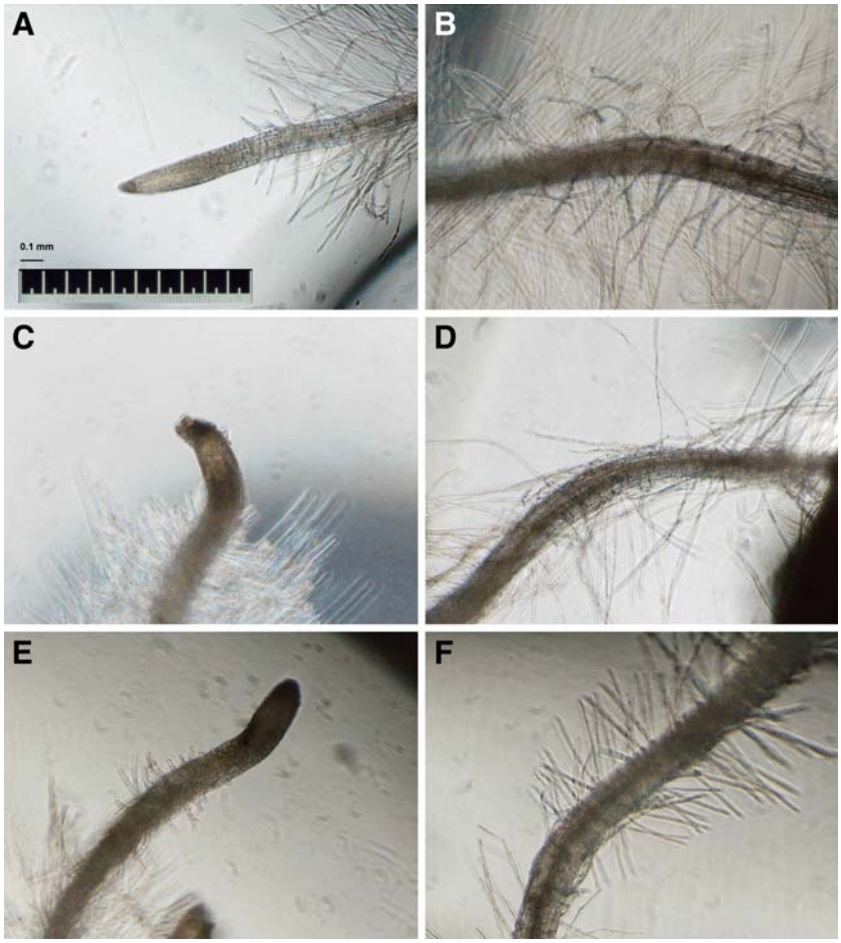

Fig. 3 Root tips (**a**, **c**, **e**) and regions of roots just behind root tips (**b**, **d**, **f**) of 10-day-old *A. thaliana* seedlings grown on half strength MS medium with no catechin (**a**, **b**), 1 mM (+)-catechin (**c**, **d**) or 1 mM (−)-catechin (**e**, **f**) for 4 days, followed by transfer to media without catechin for 4 days

no pronounced effect of pH between 5 and 8 on activity of 3.3 mM catechin on lettuce after 7 days (Fig. 9). Racemic catechin at 3.3 mM slightly stimulated growth of hypocotyls at pH 7 and 8 and roots at pH 6.

Studies in Soil There was no marked effect on growth of either Idaho fescue or junegrass by a racemic mixture of catechin in Metromix 350 potting soil at concentrations up to 10 mg/g dry weight after 13 days of growth (Fig. 10).

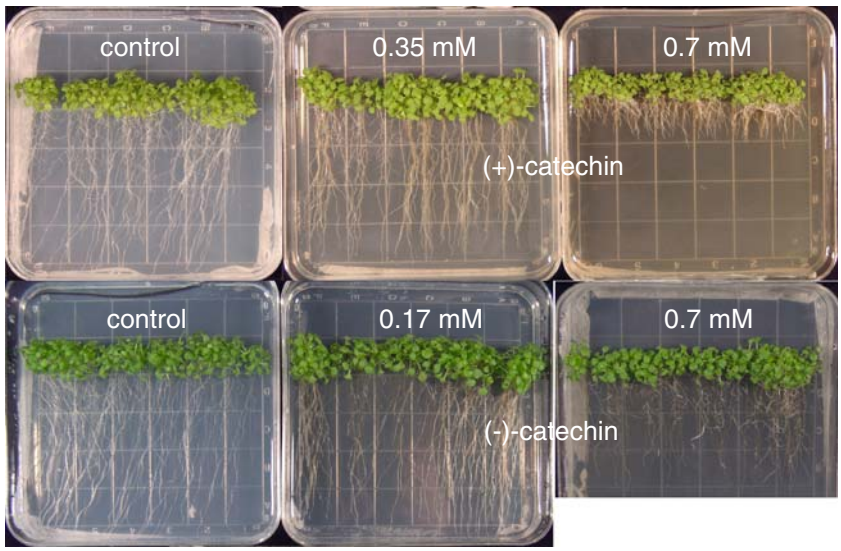

Fig. 4 Effects of different forms of catechin incorporated into the Gamborg's B5 agar media of *A. thaliana* after 14 days of growth

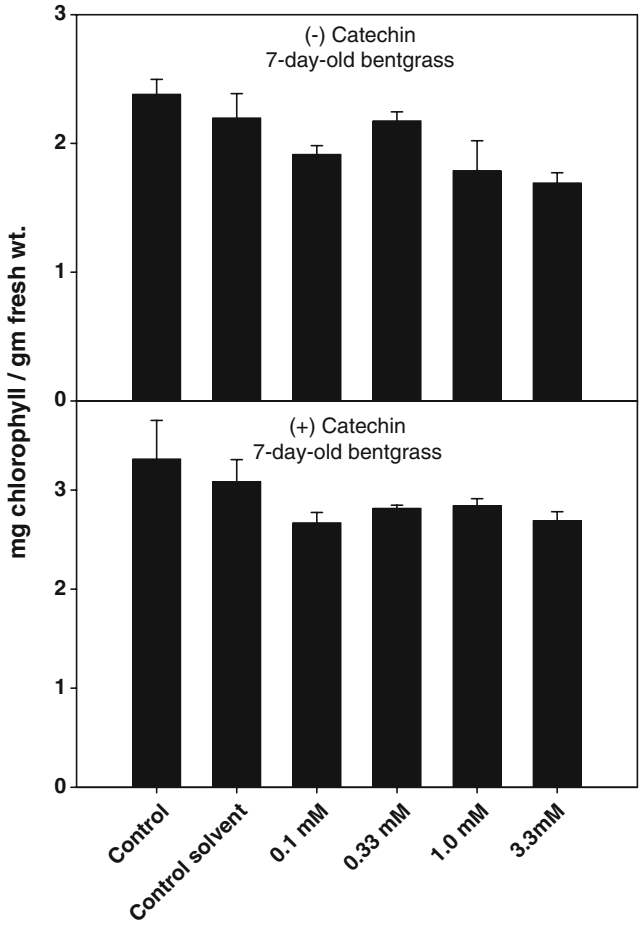

Fig. 5 Effects of different concentrations of (−)- and (+)-catechin on bentgrass chlorophyll levels after 7 days of growth in distilled water with and without the catechins. *Error bars* are ±1 SE of the mean

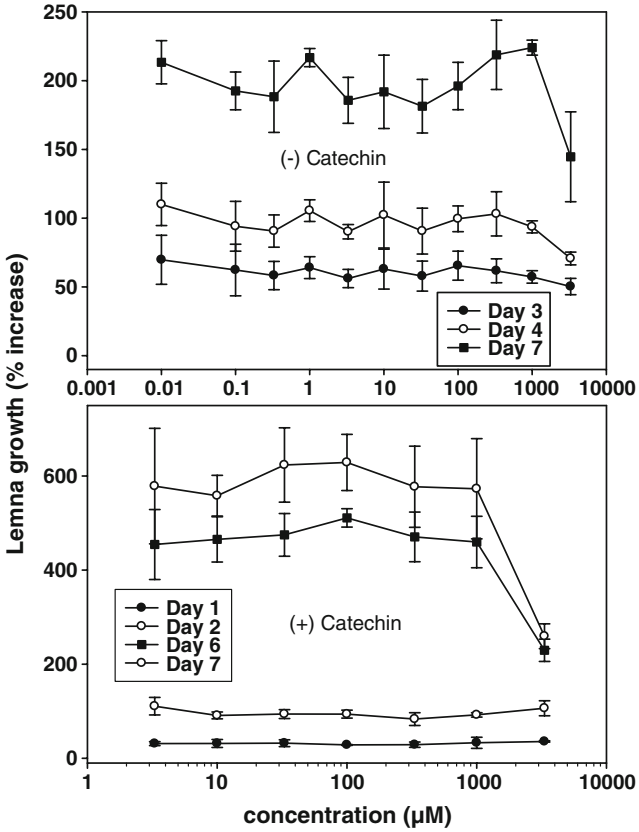

Fig. 6 Effects of different concentrations of (−)- and (+)-catechin on duckweed growth in Hoagland's solution with or without the catechins after different periods of exposure. *Error bars* are ±1 SE of the mean

Analysis of the dose–response curves using a four-parameter logistic function calculated nearly identical I_{50} values for catechin on Idaho fescue and junegrass, at 15.4 and 15.6 mg/g, respectively. The potential concentration of the combined two enantiomers in the soil at 15 mg/g dry weight is 12.5 mM. At this concentration and above, growth was significantly reduced, but the plants remained green and retained turgor.

Soils contain microflora that can convert phytotoxins to more active or less active compounds. Autoclaving can eliminate this possibility. By using autoclaved and non-autoclaved soils from Jens (Fig. 11a) and Nelson (Fig. 12a), we found little or no effect of a racemic mixture of catechin on growth of Idaho fescue plants after 10 days at concentrations below 10 mg/g dry soil (ca 8.33 mM). There was no difference between results with autoclaved and nonautoclaved soil, except at 30 and 100 mg/g of soil (ca 25 and 83 mM, respectively) in Jens and Nelson soils, respectively, and these differences were small. In non-autoclaved Nelson soil, 15 mg/g dry weight (ca 12.5 mM) of (−)-catechin had no significant effect on growth (Fig. 11A).

We considered the possibility that, over a longer time period, catechin could be converted to a more toxic compound by soil microflora, so we incubated the catechin in autoclaved or nonautoclaved moist soil for 2 weeks before planting Idaho fescue and then repeated the experiment (Figs. 11b and 12b). The relative effects of racemic catechin were similar to results from soil that had not been preincubated, but plants grew better in autoclaved than nonautoclaved soil after this 2-week incubation period in nonamended soils and those with less than 3 and 30 mg of catechin/g of soil (ca 2.5 and 25 mM, respectively) in Jens and Nelson, respectively. Idaho fescue grown in (−)-catechin-augmented Nelson soil for 12 days was stunted by 10 and 30 mg/g of soil dry weight (ca 8.33 and 25 mM, respectively; Fig. 13), but the plants were still green and turgid.

Finally, we showed that (−)-catechin is apparently as active as an antioxidant as (+)-catechin, (−)-epicatechin, and quercitin, all known to be antioxidants (Fig. 14).

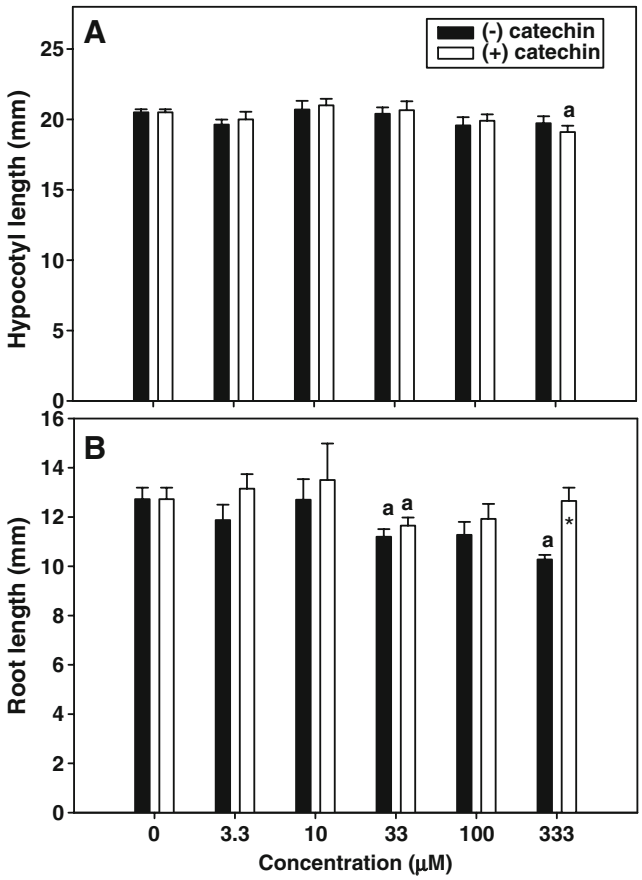

Fig. 7 Effects of different concentrations of (−)- and (+)-catechin on growth of hypocotyls (**a**) and roots (**b**) of lettuce seedlings grown in distilled water with or without the catechin after 8 days of exposure. *Error bars* are ±1 SE of the mean. Those *means with letters over them* are significantly different from the corresponding control at α=0.05, using Duncan's multiple range test. The *bar with an asterisk* in it is significantly different from the same concentration (−)-catechin treatment at α=0.05, using Duncan's multiple range test

Discussion

Our findings indicate that (+)- and (−)-catechin are not highly phytotoxic compounds in either soil-free or soil studies. Their level of phytotoxicity is much lower than that associated with many other natural phytotoxins that we have assayed in identical soil-free bioassays, such as 2β-angeloyloxy-10β-hydroxyfuranoeremophilane from the Eurasian plant *Ligularia macrophylla*, the chaparrinone-type quassinoids from *Ailanthus* spp., or the triketone leptospermone from *Leptospermum scoparium* with an IC_{50} values between 1 and 4 µM for duckweed or lettuce (Dayan et al. 1999, 2007; Cantrell et al. 2007). With respect to the novelty of the compounds, catechin is a fairly common compound, being found in plant species native to the Americas (e.g., cacao), as well as the rest of the world (e.g., tea, lentils, grapes) for which no allelopathic properties have been claimed.

The recent literature states widely varying phytotoxicity results for catechins. For example, Weir et al. (2003) report 100% mortality of Idaho fescue at 50 ppm (ca 0.17 mM), while Blair et al. (2005) only report reduced growth of this same species at 1,000 ppm (ca 3.1 mM). We found 3.3 mM (±)-catechin to have no effect on root growth of lettuce, and Tharayil et al. (2008) reported no effect of (±)-catechin on lettuce growth up to the limits of its solubility. However, Buta and Lusby (1986) reported lettuce root growth reduction of about 70% by 1 mM (+)-catechin. Some of these disparities could be due to differences in growing conditions, method of treatment, and/or biotype or variety assayed. The bioassay conditions of Buta and Lusby (1986) were not provided, making explanation of differences in results impossible. We found catechins to stunt roots of *A.*

Fig. 8 Appearance of lettuce seedlings grown with and without 3.3 mM (±)-catechin in distilled water for 8 days

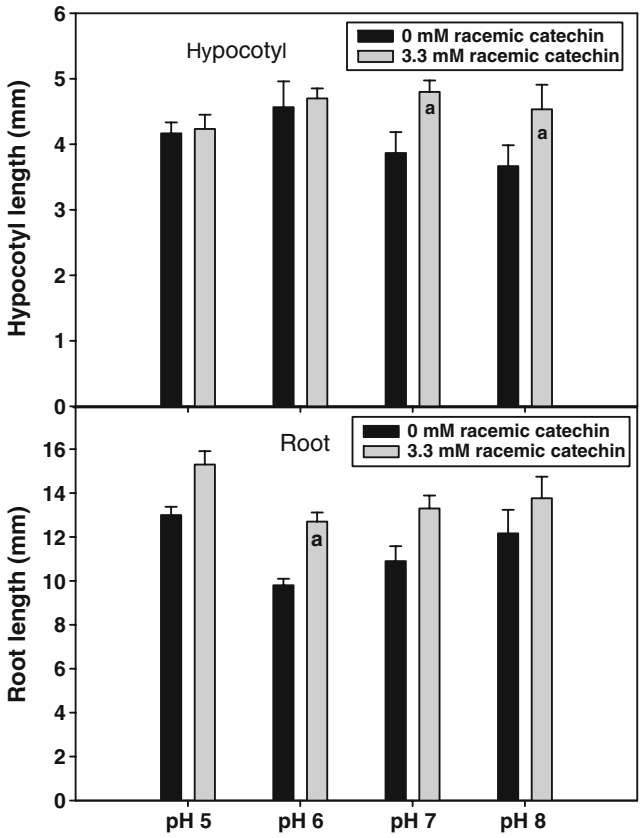

Fig. 9 Effect of 3.3 mM (±)-catechin at different pH values in distilled water (adjusted with NaOH or HCl) on growth of 8-day-old lettuce roots and cotyledons. *Error bars* are ±1 SE of the mean. Those *bars with letters* over them are significantly different from the corresponding control at α=0.05, using Duncan's multiple range test

thaliana grown continuously on agar with concentrations greater than 0.35 mM (100 μg/mL; Fig. 4). Bais et al. (2002) found 0.17 mM (+)-catechin to have little or no effect on *A. thaliana* root growth, whereas (−)-catechin was a strong inhibitor. Simões et al. (2008) found (+)-catechin to strongly inhibit root growth at concentrations above 0.35 mM when the plants were transferred into a liquid culture containing (+)-catechin that was shaken. In a similar experiment to those of Bais et al. (2002) and Simões et al. (2008), we found 1 mM catechin of either enantiomer to reduce *A. thaliana* root growth (Fig. 1), but the effects were temporary if the catechin were removed. In this experiment, the two enantiomers had similar activity, although (−)-catechin was slightly more active. In an erratum to the Bais et al. (2002) paper, the authors reported that (−)-catechin was ~1.5 to twofold more active than the positive (+) enantiomer. Our results do not support such a difference in activity.

More recently, Broeckling and Vivanco (2008) state that catechin's instability and ability to form complexes in soil confound the understanding of this compound as an allelochemical. The presence of other phenolic compounds in soil increases catechin's half-life, but it can also form a short-lived procyanidin dimer in soil, which reduces persistence and phytotoxicity of the compound (Tharayil et al. 2008). This latter paper also found (±)-catechin to be essentially nonphytotoxic to both monocotyledonous and dicotyledonous plants, even though they observed similar root browning to that attributed to necrosis by Bais et al. (2003). Tharayil et al. (2008) attributed the root browning

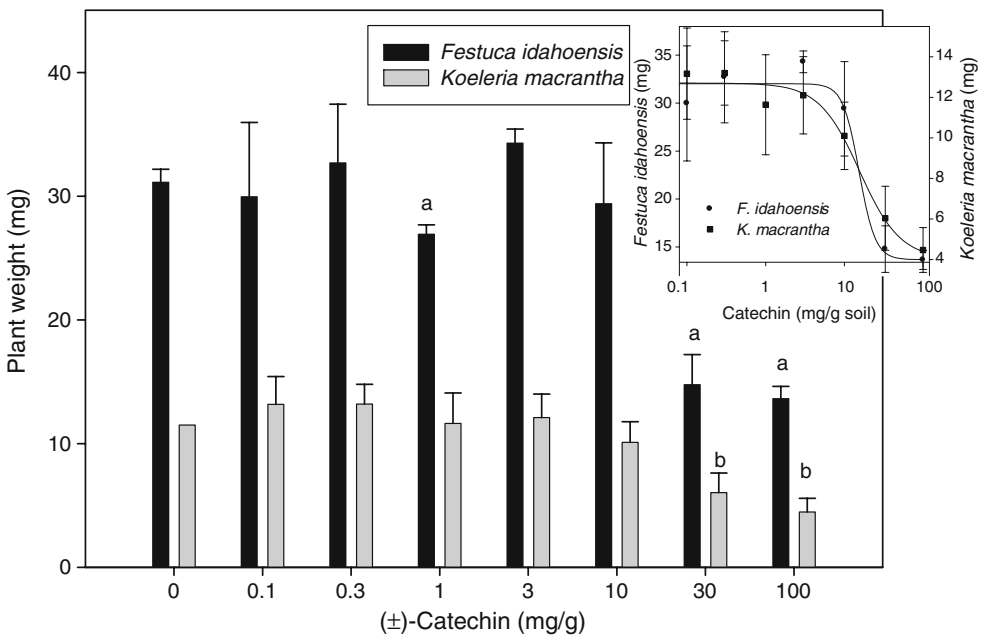

Fig. 10 Effect of different concentrations of (±)-catechin in Metromix 350 potting soil on growth of Idaho fescue (*F. idahoensis*) or junegrass (*Koeleria macrantha*) after 13 days of growth. *Error bars* are ±1 SE of the mean. Those *means with letters over them* (*a* for Idaho fescue and *b* for junegrass) are significantly different from the corresponding control at α=0.05, using Duncan's multiple range test. The *inset* shows the *R* plots of data used to generate the I_{50} values

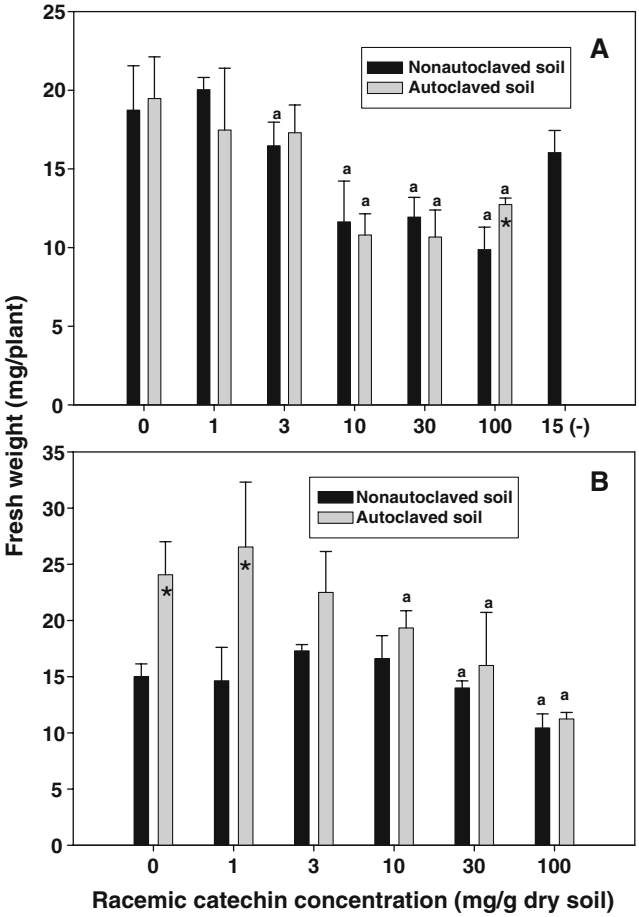

Fig. 11 Effects of different concentrations of (±)-catechin and 10 mg/g of soil (−)-catechin in autoclaved or nonautoclaved Jens soils planted with Idaho fescue immediately after amendment with the catechin (**a**) or 2 weeks after amendment (**b**). Measurements were taken 10 days after planting. *Error bars* are ±1 SE of the mean. Those means with letters over them are significantly different from the corresponding control at α=0.05, using Duncan's multiple range test. Those *bars with an asterisk* in them are significantly different from the same nonautoclaved soil treatments at α=0.05, using Duncan's multiple range test

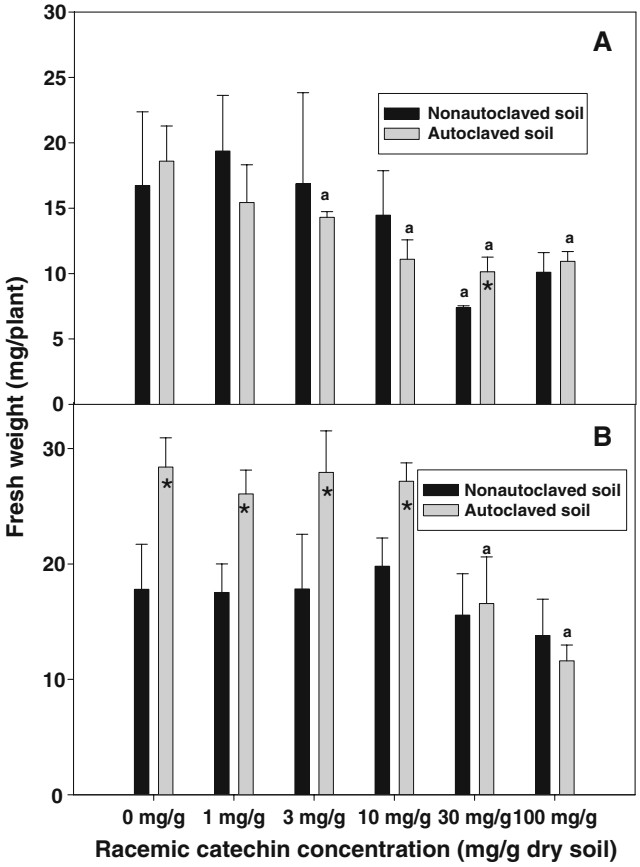

Fig. 12 Effects of different concentrations of (±)-catechin in autoclaved or nonautoclaved Nelson soils planted with Idaho fescue immediately after amendment with the catechin (**a**) or 2 weeks after amendment (**b**). Measurements were taken 10 days after planting. *Error bars* are ±1 SE of the mean. Those means with letters over them are significantly different from the corresponding control at α=0.05, using Duncan's multiple range test. Those *bars with an asterisk* in them are significantly different from the same nonautoclaved soil treatments at α=0.05, using Duncan's multiple range test

to enzyme-mediated (peroxidases or polyphenol oxidase) polymerization of catechin to brown polymers, an effect that should not contribute to toxicity. We found root browning to be somewhat reversible (Fig. 2).

Errata were published to the papers by Veluri et al. (2004), Weir et al. (2003), and Bais et al. (2002), in which the claims of differential phytotoxicity of (−) and (+) catechin and the degree of phytotoxicity of (−)-catechin were reduced, as well as the levels of catechin produced by individual plants. The same laboratory (Prithiviraj et al. 2007) then reported that low doses of catechin can stimulate growth of *A. thaliana*. They attributed this effect to induction of low levels of reactive oxygen species (ROS). Stimulatory effects of nontoxic doses of phytotoxins (hormesis) are common (Duke et al. 2006). Prithiviraj et al. (2007) also reported that these doses induced pathogen resistance, an effect observed with low doses of other chemical inducers of ROS (Duke et al. 2007).

In one of the original catechin papers (Bais et al. 2003), the mechanism of action of (−)-catechin was linked to induction of ROS. Weir et al. (2006) produced evidence that oxalate, whether produced by a plant or present in the soil, blocks the phytotoxic activity of (−)-catechin by preventing production of ROS. That ROS is involved in the mode of action of catechins as a phytotoxin is debatable because catechins are well-known antioxidants (e.g., Almajano et al. 2007). In fact, more than 1,500 papers mention catechin as an antioxidant (SciFinder® search, as of October, 2008). We are unaware of any papers that show that (−)-catechin is an antioxidant as we have (Fig. 14), but

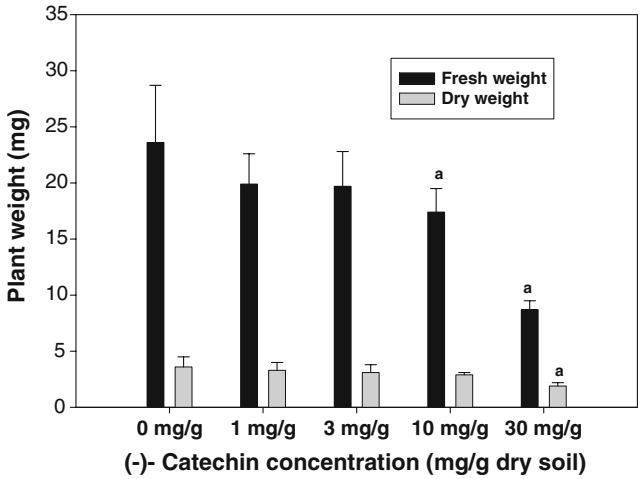

Fig. 13 Effects of (−)-catechin in Nelson soil on fresh and dry weight per plant of Idaho fescue grown for 12 days. *Error bars* are ±1 SE of the mean. Those *means with letters* over them are significantly different from the corresponding control at α=0.05, using the Duncan's multiple range test

chirality should not influence this type of activity. Antioxidants quench ROS, rather than producing them.

In soil, we found the activity of catechins to be even less than in soil-free systems, with little or no effects at theoretical concentrations in soil water as high as almost 10 mM. This approximation is probably low, as the molarity in soil would be expected to increase with time as the soil dries, provided the catechin does not degrade. At higher levels, the plants were stunted, but not killed. No one has suggested that spotted knapweed generates levels of catechin in soils that would approach 10 mM. Such high concentrations could cause growth reduction by reducing water potential. The results with regard to catechin were the same in autoclaved soils, suggesting that there is no microbial bioactivation or inactivation. Catechin is unstable in soil, especially at high pH (Furubayashi et al. 2007). Inderjit et al. (2008) also noted that (±)-catechin degrades rapidly in soil, so that the level of catechin available to cause a biological effect may be much smaller than what is actually applied. The same could be said of catechin in solution. If catechin is the causal agent of any effect, its instability would contribute to its very weak effects. Inderjit et al. (2008) found catechin to be more phytotoxic in sand or soil with organic matter mixed in it than in soil without organic matter, and they considered junegrass to be highly sensitive to (±)-catechin in soils from Montana, Romania, and India. This conclusion is in marked conflict with our results with this species. In their study, water stress increased the phytotoxicity of (±)-catechin.

In a field situation, catechin is likely to be found in combination with other potential allelochemicals. Tharayil et al. (2006, 2008) found that the individual half-lives of such compounds in soil are extended by the presence of similar compounds. Synergism of simple phenolic compounds in causing phytotoxicity in the absence of soil has not been found in carefully conducted studies (e.g., Duke et al. 1983; Gerig et al. 1989; Jia et al. 2006).

In summary, our results do not support the view that either enantiomer of catechin is involved in allelopathy of spotted knapweed. Our findings, and the fact that catechin is found in many plant species throughout the world, casts doubt on the concept that catechin is a novel weapon that enables spotted knapweed in North America to succeed against native plant species. Nevertheless, the finding by Ridenour and Callaway (2001), in greenhouse carbon addition experiments, that spotted knapweed may cause allelopathic effects on native species through an allelochemical calls for further study of this phenomenon. Rigorous bioassay-directed isolation of the responsible compound(s) might solve this mystery.

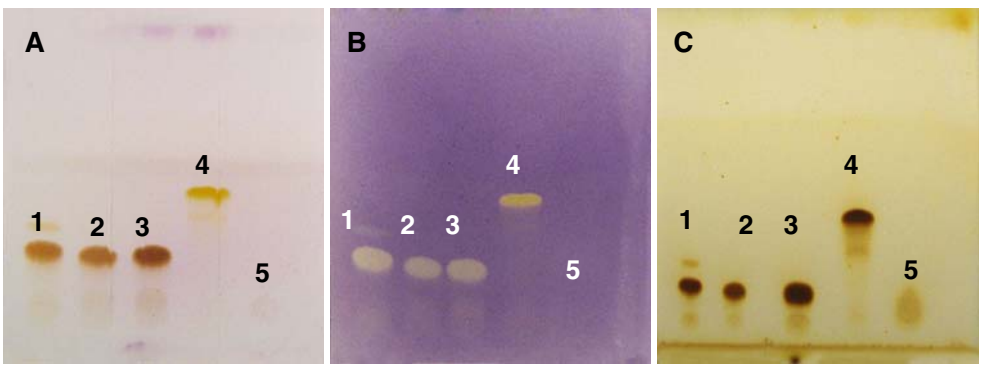

Fig. 14 Antioxidant activity of *1* (−)- epicatechin, *2* (−)-catechin, *3* (+)-catechin, *4* quercetin, *5* mannitol. **a** Plate was sprayed with anisaldehyde TLC spray reagent to visualize the compounds. **b** Plate was sprayed with 0.2% DPPH in methanol and allowed to stand for 30 min. *Yellow spots* on the purple background show the presence of antioxidant activity. **c** Plate was exposed to iodine vapor

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Essential Oil of *Artemisia scoparia* Inhibits Plant Growth by Generating Reactive Oxygen Species and Causing Oxidative Damage

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Abstract We investigated the chemical composition and phytotoxicity of the essential oil extracted from leaves of *Artemisia scoparia* Waldst. et Kit. (red stem wormwood, Asteraceae). GC/GC-MS analyses revealed 33 chemical constituents representing 99.83% of the oil. The oil, in general, was rich in monoterpenes that constitute 71.6%, with β-myrcene (29.27%) as the major constituent followed by (+)-limonene (13.3%), (*Z*)-β-ocimene (13.37%), and γ-terpinene (9.51%). The oil and β-myrcene were evaluated in a dose–response bioassay under laboratory conditions for phytotoxicity against three weeds—*Avena fatua, Cyperus rotundus*, and *Phalaris minor*. A significant reduction in germination, seedling growth, and dry matter accumulation was observed in the test weeds. At the lowest treatment of 0.07 mg/ml *Artemisia* oil, germination was reduced by 39%, 19%, and 10.6% in *C. rotundus, P. minor*, and *A. fatua*, respectively. However, the inhibitory effect of β-myrcene was less. In general, a dose-dependent effect was observed and the growth declined with increasing concentration. Among the three weeds, the inhibitory effect was greatest on *C. rotundus*, so it was selected for further studies. We explored the explanation for observed growth inhibition in terms of reactive oxygen species (ROS: lipid peroxidation, membrane integrity, and amounts of conjugated dienes and hydrogen peroxide)-induced oxidative stress. Exposure of *C. rotundus* to *Artemisia* oil or β-myrcene enhanced solute leakage, indicating membrane disintegration. There were increased levels of malondialdehyde and hydrogen peroxide, indicating lipid peroxidation and induction of oxidative stress. We conclude that *Artemisia* oil inhibits plant root growth through generation of ROS-induced oxidative damage.

Keywords *Artemisia scoparia* · Conjugated dienes · Electrolyte leakage · Essential oil · Growth inhibition · Hydrogen peroxide · Lipid peroxidation · β-Myrcene · Oxidative damage

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Introduction

Aromatic plants and their volatile oils have been in use since antiquity in flavor and fragrances, in medicines, as antimicrobial and insecticidal agents, and to repel insects and stored product pests (Bakkali et al. 2008; Batish et al. 2008). However, due to fumigant and insecticidal activity, the interest in volatile oils has increased tremendously during the last decade or so. Volatile terpenes act as pollinator attractants, provide an important defense strategy against herbivores and pathogenic fungi, play a significant role in plant–plant interactions, and depict an evolutionary relationship with their functional roles (Langenheim 1994; Batish et al. 2008). Of late, volatile oils and their constituents are being explored for weed and pest management and viewed as an important source of lead molecules (Romagni et al. 2000; Batish et al. 2004; Singh et al. 2005; Bakkali et al. 2008). It is, thus, pertinent to explore and characterize the phytotoxic properties of aromatic plants and their volatile oils.

The genus *Artemisia* (commonly wormwoods; family Asteraceae) consists of a diverse group of around 200 herb

and shrub species distributed throughout the world (Anonymous 1993). The plants are rich in volatile oils that exhibit a wide spectrum of biological activity and find extensive use in medicine (Anonymous 1993). Additionally, the volatile oil of certain *Artemisia* species (*Artemisia tridentata*, *Artemisia princeps*, and *Artemisia vulgaris*) inhibits emergence and growth of nearby plants (Weaver and Klarich 1977; Yun et al. 1993; Barney et al. 2005). The characteristic vegetation patterning around *Artemisia californica* thickets that is due to volatile terpenes is one of the classical examples of allelopathy (Muller et al. 1964). *Artemisia scoparia* (red stem wormwood) is an annual aromatic species growing wild in India. It forms monospecific strands along canals, agricultural fields, roadsides, and wastelands (Anonymous 1993). However, the role of its volatile oil in suppressing the emergence and growth of other plants in its vicinity remains largely unknown. We, therefore, extracted and characterized oil from leaves of *A. scoparia*, and assessed the phytotoxicity of both the oil and its major constituent. We selected *Avena fatua* and *Phalaris minor* (weeds of wheat crop), and *Cyperus rotundus* (a weed of rice crop) as bioassay plants with a view to test *Artemisia* oil as a weed suppressant and its potential use as a novel bioherbicide. We further explored the possible mechanism of phytotoxicity of *Artemisia* oil in terms of reactive oxygen species (ROS: lipid peroxidation, membrane integrity, and amounts of conjugated dienes and hydrogen peroxide)-induced oxidative stress.

Methods and Materials

Extraction of the Oil Volatile oil was extracted from fresh leaves of *A. scoparia* Waldst. & Kit. (red stem wormwood; hereafter *Artemisia*) by hydrodistillation using Clevenger's apparatus. For this, during the second week of June 2007 (summer season), leaves were collected from *Artemisia* plants growing in wastelands around Chandigarh.

Leaves (250 g) were chopped and mixed with distilled water (1 l) in a round bottom flask (2 l) and boiled for 3 h. The oil was collected from the nozzle of the condenser, dried under sodium sulfate, and stored at 4°C for identification and bioassay. The oil was clear yellow in color with a yield of 0.17% (*v*/*w*, fresh weight basis).

Oil Analysis The extracted oil was analyzed by gas chromatography (GC) and gas chromatography–mass spectroscopy (GC-MS) as per Singh et al. (2009).

GC was done on a Shimadzu GC-17A gas chromatograph equipped with a flame ionization detector (FID) and DB-5 column (60 m×0.25 mm, i.d., film thickness 0.25 μm). Helium (He) at a split ratio 1:20 and a flow rate 1 ml min^{-1} was used as carrier gas. The injector and detector temperature were set at 250°C and 280°C, respectively. Initially, the oven temperature was 50°C, held isothermally for 2 min, then increased to 260°C at the rate of 4°C min^{-1}, and finally held at 260°C for 3 min. The relative amount of different constituents was determined by computerized peak area normalization based upon three injections of the oil, without any correction factor. Peaks were compared with data from GC-MS.

GC-MS analysis was performed on a Shimadzu QP 2010 mass spectrophotometer equipped with fused silica (SGE BP 20) capillary column (30 m×0.25 mm, i.d., 25 μm film thickness). He at a split ratio of 1:50 and a linear velocity of 38.5 cm s^{-1} was used as carrier gas. The injector and detector temperature were set at 220°C and 250°C, respectively. The temperature was programmed from 70°C (held isothermally for 4 min) to 220°C at the rate of 4°C min^{-1} and held at 220°C isothermally for 5 min. Mass spectral range was recorded from *m/z* 40 to 600 amu. The identification of different constituents was based on: (1) the comparison of their retention times with those of pure reference samples from Sigma-Aldrich (St. Louis, MO, USA), Fluka (Buchs, Switzerland), Acros (Geel, Belgium), AlfaAeser (Ward Hill, MA, USA), and TCI (Tokyo, Japan); (2) co-elution with available authentic standards; (3) comparison of retention indices (RI) with reference to a homologous series of *n*-alkanes (C_7-C_{30}; Supelco, Bellefonte, PA, USA); and (4) computer matching of mass spectra by using library search system HP-5872 (Hewlett-Packard) and consulting data bases of Wiley 275 and NBS 75K Libraries (McLafferty 1989), NIST 98 (Stein 1990), and compilation by Adams (1995).

Procurement of Materials For laboratory bioassay, seeds of *A. fatua*, *C. rotundus*, and *P. minor* were collected locally from agricultural fields on the outskirts of Chandigarh. These were surface-sterilized with sodium hypochlorite (1%, *w*/*v*) for 2 min, washed under running tap water (for 5 min) followed by distilled water and stored for further use. β-Myrcene (the major component of *Artemisia* oil) of technical grade (purity 90%) was used in the bioefficacy experiments and purchased from AlfaAesar (Ward Hill, MA, USA). All other chemicals were of analytical grade and purchased from the best available sources (Sisco Research Laboratory, Mumbai, India; Loba-Chemie, Mumbai; Sigma-Aldrich, St. Louis, MO, USA).

Laboratory Bioassay *Artemisia* oil and β-myrcene were evaluated for phytotoxicity in a dose–response manner under laboratory conditions. Seeds of test plants were imbibed in distilled water for 16 h. Pre-imbibed seeds (15 of *A. fatua* or 25 of *C. rotundus* and *P. minor*) were placed in 15-cm diameter Petri dishes on Whatman No. 1 filter paper moistened with 7 ml of distilled water. Different amounts (0.5–5.0 μl) of *Artemisia* oil or β-myrcene were loaded onto

the inner side of the lid of the Petri dish to obtain concentrations of 0.07, 0.14, 0.35, and 0.70 mg/ml. After treatment, dishes were sealed with Parafilm®. A set of Petri dishes without oil or β-myrcene served as a parallel control. For each treatment, including control, five Petri dish replicates were maintained in a randomized block design. All dishes were kept in a growth chamber set at 25±2°C temperature, 16/8 h L/D photoperiod with photosynthetic photon flux density of ~225 μmole m^{-2} s^{-1} and a relative humidity of nearly 80%. After 1 week, the number of seeds that germinated was counted and the length (from tip of root to tip of shoot) and dry weight (by oven drying at 60°C for 48 h) of emerged seedlings were measured. Among the three weeds, the inhibitory effect was the greatest on *C. rotundus*, so it was selected for further biochemical studies by exploring the induction of oxidative damage as a possible mechanism of phytotoxicity. Roots of *C. rotundus* were excised and kept at 4°C prior to further studies/analyses.

Determination of Cell Survivability Cell survivability was determined spectrophotometrically by using 2,3,5-triphenyl tetrazolium chloride (TTC) as per Singh et al. (2002). It provides an indirect method of measurement of cell respiration since TTC absorbs electrons from the respiratory chain (Batish et al. 2007a). Absorbance was read at 530 nm and the values expressed with respect to control.

Effect on Membrane Integrity Effect of *Artemisia* oil or β-myrcene was studied on membrane integrity (an indicator of cellular damage) in terms of ion leakage from the roots of *C. rotundus* by measuring conductivity of the bathing medium (Duke and Kenyon 1993). Roots (200 mg) were incubated in 5 ml of 1 mM MES buffer (2-[N-morpholino] ethanesulphonic acid sodium salt, pH 6.5) containing 2% sucrose with *Artemisia* oil or β-myrcene (0.14 and 0.35 mg/ml; selected on the basis of the laboratory bioassay) dissolved in Tween-80. A parallel set up without *Artemisia* oil or β-myrcene, but containing MES buffer and Tween-80 only, was maintained as control. The conductivity of the bathing medium containing *C. rotundus* roots with or without treatment was measured with a conductivity meter (ECOSCAN CON5, Eutech Instuments Pte. Ltd., Singapore) at regular intervals in dark (0, 1, 2, 4, 8, 12, 16, 18, and 20 h) followed by exposure to light for a further 10 h (i.e., at 22, 24, 26, 28, and 30 h). The maximum electrolyte leakage from root tissue was determined by boiling the roots for 15 min. The experiment was repeated twice with five replicates and the data are presented as means.

Lipid Peroxidation Lipid peroxidation was measured in terms of malondialdehyde (MDA) and conjugated dienes content. MDA, a major thiobarbituric acid reactive species, was determined as per Heath and Packer (1968). Roots (100 mg) of *C. rotundus* were homogenized in 5 ml of trichloroacetic acid (TCA, 0.1%, *w*/*v*) in a pre-chilled pestle and mortar and centrifuged at 10,000×*g* for 10 min. One milliliter of the supernatant was added to 4 ml of thiobarbituric acid (0.5%, *w*/*v*, in 20% TCA). The mixture was heated at 95°C for 30 min, cooled over ice, and then centrifuged at 10,000×*g* for 10 min. The absorbance of the supernatant was read at 532 nm and corrected for non-specific absorbance at 600 nm. MDA content was determined by using the extinction coefficient (ε) of 155 mM cm^{-1} and expressed as nmol g^{-1} FW (fresh weight).

Conjugated dienes content was determined by homogenizing roots (100 mg) of *C. rotundus* in 5 ml of 95% (*v*/*v*) ethyl alcohol (Singh et al. 2007). The mixture was centrifuged at 10,000×*g* for 10 min, and the absorbance was read at 234 nm. The content of conjugated dienes was determined by using an extinction coefficient of 26.5 mM cm^{-1}, and expressed as μmol g^{-1}FW.

Determination of Hydrogen Peroxide (H_2O_2) Content

Roots (100 mg) of *C. rotundus* were homogenized with 5 ml of 0.1% TCA (*w*/*v*) in a pre-chilled pestle and mortar and centrifuged at 12,000×*g* for 15 min (Singh et al. 2007). To 0.5 ml of supernatant, 0.5 ml of phosphate buffer (pH 7), and 1 ml of 1 M potassium iodide were added. The absorbance of reaction mixture was measured at 390 nm. H_2O_2 content was determined by using an extinction coefficient (ε=0.28 μM cm^{-1}) and expressed as nmol g^{-1}FW.

Statistical Analyses The relative amount of different constituents of *Artemisia* oil determined by GC/GC-MS analyses was based upon three injections of the oil. The dose–response laboratory bioassay was conducted in a randomized block design with five replications, each comprised of a single Petri dish. Five independent tissue samples were used as replicates for each analysis. The data are presented as mean±SE and analyzed by one-way ANOVA followed by the comparison of mean values using post-hoc Tukey's test at $P \leq 0.05$.

Results and Discussion

Composition of Essential Oil from Young Leaves GC and GC-MS analyses of *Artemisia* oil revealed it to be a mixture of monoterpenoids, sesquiterpenes, aromatic compounds, aliphatic esters, ketones, and alcohols, which eluted between 4.16 to 40.29 min. A total of 33 chemical constituents representing 99.83% of the essential oil, were identified (Table 1). Of these, β-myrcene (29.27%) was the

main constituent, followed by (+)-limonene (13.3%), (*Z*)-*β*-ocimene (13.37), *γ*-terpinene (9.51%), and acenaphthene (17.8%). These together constituted ~86% of the oil (Table 1). The oil, in general, was rich in monoterpenes (71.6%), while the other compounds constituted 28.23%. The composition of the characterized oil was in sharp contrast to earlier reports. Earlier, 1-phenyl-penta-2,4-diyne, *β*-pinene, limonene, and (*E*)-*β*-ocimene (Safaei-Ghomi et al. 2005), or methyl eugenol (Basher et al. 1997), or thujone, camphor, and 1,8-cineole (Mirjalili et al. 2007) were identified as the main constituent of the oil from aerial parts of *A. scoparia*. These components were either absent or present in small amounts in the present study. Nevertheless, our observations are parallel to earlier studies that reported *β*-myrcene as the main constituent in the oil extracted from leaves and residues of *A. scoparia* (Singh et al. 2008, 2009). The observed variations in oil composition among those reported in literature are apparently largely due to differences in growth stages, plant parts, harvesting time, variations in edaphic and climatic factors, and geographical region (Batish et al. 2006b, 2008).

Effect on Germination and Early Growth A significant reduction in germination of test weeds was observed in response to *Artemisia* oil and *β*-myrcene (Table 2). At the lowest treatment of 0.07 mg/ml *Artemisia* oil, germination was reduced by 39%, 19%, and 10.6% in *C. rotundus*, *P. minor*, and *A. fatua* (Table 2). The inhibitory effect of *β*-myrcene was less. Inhibitory effects were greatest on *C. rotundus*, followed by *P. minor* and then *A. fatua*. In general, a gradual decline in germination with increasing concentration was observed, thus indicating a dose-dependent effect.

Table 1 Chemical constituents identified by GC-MS in the essential oil extracted from leaves of *Artemisia scoparia*

Retention time (min)	Compound[a]	RI[b]	Percent amount[c]	Identification
4.16	β-Myrcene	1161	29.27	co-GC, RI, MS
4.46	α-Terpinene	1181	0.37	co-GC, RI, MS
4.82	(+)-Limonene	1201	13.30	co-GC, RI, MS
5.01	1,8-Cineole	1210	0.05	co-GC, RI, MS
5.59	(Z)-β-Ocimene	1231	13.37	co-GC, RI, MS
5.82	γ-Terpinene	1244	9.51	co-GC, RI, MS
5.98	(E)-β-Ocimene	1250	2.34	co-GC, RI, MS
6.44	p-Cymene	1269	0.71	co-GC, RI, MS
6.72	α-Terpinolene	1280	0.56	co-GC, RI, MS
7.39	(Z)-3-Hexenol	1307	0.05	co-GC, RI, MS
7.71	(Z)-3-Hexenyl acetate	1318	0.15	RI, MS
9.27	Allo-ocimene	1370	0.14	co-GC, RI, MS
12.84	(Z)-3-Hexenyl isobutyrate	1484	0.08	RI, MS
14.77	(−)-Linalool	1547	0.07	co-GC, RI, MS
15.83	α-Caryophyllene	1582	0.77	co-GC, RI, MS
16.25	Terpinen-4-ol	1596	0.06	RI, MS
18.22	Citronellyl acetate	1658	0.42	co-GC, RI, MS
18.81	γ-Curcumene	1681	0.04	RI, MS
19.13	α-Terpineol	1692	0.06	co-GC, RI, MS
20.06	Neryl acetate	1723	0.01	co-GC, RI, MS
20.94	Geranyl acetate	1753	0.96	co-GC, RI, MS
21.19	trans-Pinocarvyl acetate	1762	0.28	RI, MS
21.27	β-Citronellol	1765	0.10	co-GC, RI, MS
25.98	cis-Jasmone	1942	0.15	co-GC, RI, MS
26.82	Caryophyllene oxide	1966	0.35	co-GC, RI, MS
27.93	Methyl eugenol	2007	0.40	co-GC, RI, MS
28.09	2,4-Pentadiynyl-benzene	2022	1.49	RI, MS
28.66	trans-Nerolidol	2036	0.17	co-GC, RI, MS
30.62	(−)-Spathulenol	2116	0.39	RI, MS
31.84	Eugenol	2167	5.52	co-GC, RI, MS
33.35	Acenaphthene	2229	17.80	co-GC, RI, MS
37.76	Acetylisoeugenol	2442	0.49	RI, MS
40.29	Isomenthyl acetate	2540	0.40	RI, MS

[a] Compounds presented in order of elution from the SGE-BP20 capillary column
[b] Retention index (RI) relative to *n*-alkanes (C_7-C_{30}) on the SGE-BP20 capillary column
[c] Percentage based on FID peak area normalization

Table 2 Effect of essential oil from leaves of *Artemisia scoparia* and its major monoterpene (β-myrcene) on percent germination of test plants

Conc. (mg/ml)	*Artemisia* oil			β-Myrcene		
	Avena fatua	*Phalaris minor*	*Cyperus rotundus*	*Avena fatua*	*Phalaris minor*	*Cyperus rotundus*
0 (Control)	97.0±1.7 a	100.0±0.0 a	95.2±1.12 a	97.0±1.2 a	100.0±0.0 a	95.2±1.12 a
0.07	86.7±2.01 b	81.0±1.03 b	58.3±1.12 b	96.0±2.3 a	94.4±3.11 b	78.3±2.49 b
0.14	65.0±1.87 c	63.2±0.57 c	23.2±0.89 c	82.7±1.81 b	80.3±3.67 c	48.2±2.56 c
0.35	28.3±1.90 d	45.0±1.34 d	7.3±0.45 d	69.7±1.32 c	64.0±1.23 d	26.7±1.97 d
0.70	10.0±0.55 e	0 e	0 e	44.3±1.9 d	40.3±2.51 e	0 e

Within a column, means with common letters are not significantly different at $P \leq 0.05$, according to Tukey's test

Artemisia oil and β-myrcene significantly reduced radicle growth and seedling dry weight of test weeds (Tables 3 and 4). Radicle elongation of *A. fatua* was reduced by ~47% and 26% in response to 0.14 mg/ml of *Artemisia* oil and β-myrcene, respectively. At 0.35 mg/ml concentration of *Artemisia* oil and β-myrcene, radicle growth was reduced in the range of 37–94% and 12–63%, respectively (Table 3). Upon exposure to 0.35 mg/ml *Artemisia* oil, ~94% reduction in root length was observed. Parallel to root growth, a significant reduction in dry weight of the emerged seedlings also was observed in response to both *Artemisia* oil and β-myrcene (Table 4).

Reduction in germination and radicle growth by volatile terpenes from *A. scoparia* is not surprising. Earlier, the volatile oils from *A. tridentata*, *A. princeps*, *A. vulgaris*, and *A. scoparia* were reported to inhibit emergence and growth of associated vegetation (Weaver and Klarich 1977; Yun et al. 1993; Barney et al. 2005; Singh et al. 2008). Several studies have documented that the oil from aromatic plants and volatile terpenes are potent inhibitor of seed germination and root elongation. For example, Muller et al. (1964) demonstrated that volatile oils from *Salvia leucophylla* and *Artemisia californica* reduced the growth of associated plants, thus resulting in characteristic vegetational patterning. Batish et al. (2004, 2006a, b) reported a growth inhibitory effect of volatile oil from *Eucalyptus citriodora* on the emergence, radicle, and early seedling growth of several plants.

Phytotoxicity of volatile oils and terpenes also has been implicated as one of the possible reasons for successful colonization by invasive weeds (Kong et al. 1999; Barney et al. 2005; Ens et al. 2008). Kong et al. (1999) characterized a number of allelopathic constituents from the essential oil of *Ageratum conyzoides* that possibly are responsible for its invasive nature. Similarly, Barney et al. (2005) identified a number of volatile allelochemicals from the fresh leaves of invasive weed mugwort (*A. vulgaris*). These workers showed that nine of the identified monoterpenes act synergistically, impart phytotoxicity to the weed, and thus help in successful colonization, and habitat invasion (Barney et al. 2005). Recently, volatile sesquiterpenes (β-maaliene, α-isocomene, β-isocomene, δ-cadinene, 5-hydroxycalamenene, and 5-methoxycalamenene) that emanate from roots of the invasive plant bitou bush (*Chrysanthemoides monilifera* spp. *rotundata*) were shown to inhibit the seedling growth of associated native vegetation, and thus possibly help in successful invasion in the introduced sites (Ens et al. 2008). Additionally, several constituent terpenes are potent inhibitors of seed germination and seedling growth. These include: 1,4-and 1,8-cineole (Romagni et al. 2000), citronellal, citronellol, linalool (Singh et al. 2002, 2006b), α-pinene (Abrahim et al. 2000; Singh et al. 2006a), and limonene (Abrahim et al. 2000).

Inhibition of germination and root growth by oil from foliage of *A. scoparia* suggests that under natural conditions these volatile terpenes may emanate from the plant, enter the soil, and may be involved in suppression of associated vegetation, thus resulting in the formation of its monospecific strands. The soil from underneath the *A. scoparia* plants

Table 3 Effect of essential oil from leaves of *Artemisia scoparia* and its major monoterpene (β-myrcene) on the radicle length (cm) of test plants

Conc. (mg/ml)	*Artemisia* oil			β-Myrcene		
	Avena fatua	*Phalaris minor*	*Cyperus rotundus*	*Avena fatua*	*Phalaris minor*	*Cyperus rotundus*
0 (Control)	4.15±0.12 a	6.51±0.14 a	1.75±0.09 a	4.15±0.12 a	6.51±0.14 a	1.75±0.09 a
0.07	2.38±0.22 b	4.01±0.10 b	0.79±0.05 b	3.41±0.03 b	5.35±0.89 b	0.90±0.04 b
0.14	1.94±0.11 c	2.77±0.07 c	0.42±0.03 c	3.08±0.1 b	4.04±0.02 c	0.49±0.01 c
0.35	1.13±0.08 d	1.09±0.03 d	0.11±0.06 d	2.63±0.03 c	2.31±0.06 d	0.21±0.02 d
0.70	–[a]	–	–	1.82±0.03 d	1.02±0.13 e	–

Within a column, means with common letters are not significantly different at $P \leq 0.05$, according to Tukey's test
[a] No values, since no germination

Table 4 Effect of essential oil from leaves of *Artemisia scoparia* and its major monoterpene (β-myrcene) on seedling weight (mg/seedling) of test plants

Conc. (mg/ml)	*Artemisia* oil			β-Myrcene		
	Avena fatua	*Phalaris minor*	*Cyperus rotundus*	*Avena fatua*	*Phalaris minor*	*Cyperus rotundus*
0 (Control)	0.67±0.05 a	1.07±0.03 a	0.19±0.04 a	0.67±0.05 a	1.07±0.04 a	0.19±0.04 a
0.07	0.49±0.03 b	0.73±0.02 b	0.14±0.02 b	0.51±0.03 b	0.84±0.02 b	0.17±0.01 a
0.14	0.28±0.03 c	0.53±0.01 c	0.10±0.05 c	0.40±0.14 c	0.66±0.01 c	0.14±0.02 c
0.35	0.17±0.01 d	0.28±0.01 d	–	0.34±0.02 d	0.44±0.03 d	0.11±0.01 d
0.70	–[a]	–	–	–	–	–

Within a column, means with common letters are not significantly different at $P \leq 0.05$, according to Tukey's test
[a] No values, since no germination

contains a mixture of mono-and sesquiterpenes. The major ones include limonene, eugenol, 1,8-cineole, ocimene, and caryophyllene oxide (data not presented), and all these are potent germination inhibitors (Singh et al. 2003).

Although the mode of inhibitory action of the oils remains somewhat unclear, volatile oils and monoterpenes inhibit cell division and induce structural breakdown and decomposition in roots (Romagni et al. 2000; Nishida et al. 2005; Singh et al. 2006a, b). Vaughn (1991) reported that essential oil from *Cinnamomum zeylanicum* and *Thymus vulgaris* inhibit the sprout growth in potato by killing meristematic cells. Batish et al. (2007b) reported that volatile oil from *E. citriodora* inhibited root growth by suppressing mitotic activity. Earlier, Scrivanti et al. (2003) reported that essential oil from *Tagetes minuta* and ocimene (a constituent monoterpene) induce severe lipid peroxidation and membrane disintegration in maize. Recently, it was demonstrated that α-pinene, a volatile monoterpene, inhibits root growth of *Cassia occidentalis* by inducing oxidative stress measured in terms of increased lipid peroxidation, H_2O_2 accumulation, and membrane disintegration (Singh et al. 2006a).

Effect on Cell Survivability *Artemisia* oil caused a significant reduction in cellular survivability (measured as % TTC reduction) in roots of test weeds (Table 5). The cellular survivability decreased 12–24% and 6–22% in response to 0.07 mg/ml of *Artemisia* oil and β-myrcene, respectively. It declined further with increasing concentrations. In general, inhibition in cellular survivability was the greatest in *C. rotundus* followed by *A. fatua,* and *P. minor*, respectively (Table 5).

Cell viability provides an indirect measurement of cell respiration (Batish et al. 2007a). In viable (respiring) tissue, TTC absorbs electrons from the mitochondrial transport chain, get reduced, and thus correlates positively with respiratory activity (Batish et al. 2007a). The decrease in cellular survivability (thus respiration) upon exposure to *Artemisia* oil or β-myrcene implies an interference with energy metabolism involved in synthesis of macromolecules, thus resulting in reduced growth. These observations are in agreement with earlier studies (Abrahim et al. 2000; Singh et al. 2005). Abrahim et al. (2000) demonstrated that monoterpenes, due to their high lipophilicity, act as uncouplers of oxidative phosphorylation, and imbalance the cellular energy levels.

Effect on Membrane Integrity Membrane disruption by monoterpenoids has been suggested as one of the mechanisms for fungicidal and bactericidal activity (Singh et al. 2006a); we, therefore, studied the effect of *Artemisia* oil and β-myrcene on membrane integrity. *Artemisia* oil and β-myrcene caused a significant ion leakage from roots of *C. rotundus* as indicated by increased electrical conductivity of the bathing medium (MES buffer). The ion leakage

Table 5 Effect of essential oil from leaves of *Artemisia scoparia* and its major monoterpene (β-myrcene) on cellular survivability (%) in test plants

Conc. (mg/ml)	*Avena fatua*	*Phalaris minor*	*Cyperus rotundus*	*Avena fatua*	*Phalaris minor*	*Cyperus rotundus*
0 (Control)	100.0±0.0 a	100.0±0.0 a	100.0±0.0a	100.0±0.0 a	100.0±0.0 a	100.0±0.0 a
	Artemisia oil			β-Myrcene		
0.07	85.9±2.34 b	88.1±2.07 b	76.8±1.84 b	93.5±1.18 b	79.2±3.9 b	78.2±2.09 b
0.14	74.2±2.24 c	66.7±1.58 c	52.3±2.72 c	70.8±2.65 c	59.0±1.17 c	52.7±2.32 c
0.35	43.4±1.47 d	26.6±1.37 d	23.9±3.45 d	53.0±2.03 d	34.5±1.35 d	21.0±1.34 d
0.70	–[a]	–	–	27.7±3.04e	11.9±0.20 e	–

Within a column, means with common letters are not significantly different at $P \leq 0.05$, according to Tukey's test
[a] No values, since no germination

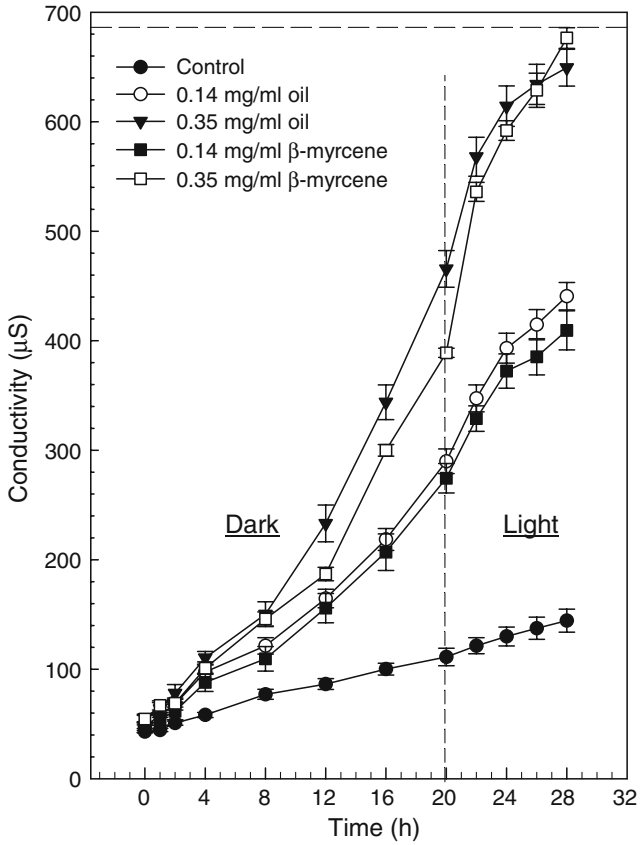

Fig. 1 Effect of *Artemisia* oil and *β*-myrcene on membrane integrity measured as electrolyte leakage from roots of *Cyperus rotundus*. The *dotted line* at the top represent maximum conductivity (685.2 µS) measured upon boiling of plant tissue. *Vertical line* marks the time period after which the samples were exposed to light

increased with time up to 20 h in the dark and then for another 8 h in light (i.e., up to 28 h). After 28 h, conductivity of the bathing medium in response to 0.14 and 0.35 mg/ml of *Artemisia* oil or *β*-myrcene was in the range of 60–64% and 95–99%, respectively, of the maximum leakage (685.2 µS; observed upon boiling the root tissue) (Fig. 1).

The increased conductivity of the bathing medium indicates cellular membrane disruption resulting in excessive solute leakage. These results are in agreement with earlier reports that essential oil and the monoterpenes (citronellal, α-pinene, and (+)-pulegone) cause ion leakage from plant tissue (Maffei et al. 2001; Singh et al. 2006a, b). Maffei et al. (2001) demonstrated that essential oil from *Mentha*×piperata and its pure component, (+)-pulegone, induced membrane depolarization in cucumber roots that resulted in an altered flux of ions across the membrane. Dayan et al. (2000) opined that a loss in membrane integrity relates to induction of oxidative stress. In fact, enhanced electrolyte leakage is an indicator of membrane damage due to generation of reactive oxygen species (Singh et al. 2006a). Montillet et al. (2005) reported that membrane disruption occurs due to peroxidation of polyunsaturated fatty acids or lipids in biomembranes and results in formation of byproducts such as malondialdehyde. Thus, we measured the MDA content, amount of conjugated dienes, and hydrogen peroxide content (as indicators of oxidative stress) in response to *Artemisia* oil or *β*-myrcene exposure.

Effect on Lipid Peroxidation Exposure to *Artemisia* oil significantly (at $P<0.05$) enhanced the MDA content in roots of *C. rotundus* (Table 6). It increased by 1.24- to 2.3-fold upon exposure to *Artemisia* oil or *β*-myrcene over that of control (Table 6). Increased content of MDA (a major thiobarbituric acid reactive substance) indicates enhanced lipid peroxidation and damage to membranes. These observations are in conformity with earlier reports that essential oil and monoterpenes induce membrane damage, and thus inhibit root growth (Scrivanti et al. 2003; Zunino and Zygadlo 2004; Singh et al. 2006a). The volatile oil from *Tagetes minuta* and *Schinus areira* and its monoterpenes (ocimene, ocimenone, α-pinene, 1,8-cineole, thymol, geraniol, menthol, and camphor) have been reported to inhibit maize growth by causing lipid peroxidation (Scrivanti et al. 2003; Zunino and Zygadlo 2004). Singh et al. (2006a) demonstrated that α-pinene induced severe lipid peroxidation in roots of *C. occidentalis*, damaged cellular membranes, and inhibited root growth.

Table 6 Effect of *Artemisia* oil and *β*-myrcene on lipid peroxidation (as MDA content), conjugated diene and hydrogen peroxide (H_2O_2) content in roots of *Cyperus rotundus*

Conc. (mg/ml)	MDA content (nmol/g FW)		Conjugated diene content (µmol/g FW)		Hydrogen peroxide content (nmol/g FW)	
	Artemisia oil	*β*-Myrcene	*Artemisia* oil	*β*-Myrcene	*Artemisia* oil	*β*-Myrcene
0 (Control)	15.5±0.20 a	15.5±0.20 a	7.8±0.12 a	7.8±0.12 a	61.8±2.15 a	61.8±2.15 a
0.07	20.1±0.60 b	19.2±1.09 b	7.6±0.10 a	7.1±0.13 b	80.8±3.00 b	74.8±2.90 b
0.14	25.7±0.90 c	19.6±1.09 b	5.4±0.09 b	6.8±0.15 b	96.0±4.80 c	87.5±1.80 c
0.35	35.3±1.03 d	26.5±0.60 c	3.2±0.07 c	3.0±0.14 c	107.0±4.20 d	93.1±3.10 c

Within a column, means with common letters are not significantly different at $P\leq0.05$, according to Tukey's test

Enhanced MDA content due to lipid peroxidation indicates an induction of ROS-generated oxidative stress.

Membrane damage upon exposure to *Artemisia* oil or β-myrcene was confirmed further by a significant decline in conjugated dienes content (Table 6). Upon exposure to ≥0.14 mg/ml of *Artemisia* oil or β-myrcene, the conjugated dienes content in roots of *C. occidentalis* decreased by 21–59% and 9–13%, respectively (Table 6). Decreased conjugated dienes further indicate a ROS-induced damage to biological membranes (Singh et al. 2007).

Effect on H_2O_2 Content Parallel to MDA accumulation, the amount of H_2O_2 increased significantly in roots of *C. rotundus* upon exposure to *Artemisia* oil or β-myrcene (Table 6). *Artemisia* oil and β-myrcene exposure increased H_2O_2 by 1.3- to 1.7-fold and 1.2- to1.5-fold, respectively, compared with control. At 0.35 mg/ml of *Artemisia* oil or β-myrcene, H_2O_2 content increased by 1.5- and 1.7-fold, respectively, over control (Table 6). Accumulation of H_2O_2 in *C. rotundus* roots further enhances lipid peroxidation, thus resulting in increased oxidative stress, and leading to disruption of metabolic activities in the cell. In general, H_2O_2 hinders the activity of ~SH group containing enzymes, impairs the photosynthetic activity in chloroplasts, and thereby reduces plant growth (Takeda et al. 1995). Among the different ROS produced in cells in response to environmental stresses, H_2O_2 acts as a signaling molecule. At low concentrations, it aids in cellular defense, and provides tolerance against stress, whereas at high concentrations, it induces cellular damage (Stone and Yang 2006). H_2O_2 is removed by antioxidant enzymes such as catalases, and ascorbate peroxidases, which were not studied in the present study.

In summary, the present study concludes that *Artemisia* oil inhibits germination and plant root growth through generation of ROS-induced oxidative stress associated with membrane disruption, enhanced lipid peroxidation, and accumulation of H_2O_2. Whether this induction of oxidative stress in response to *Artemisia* oil was accompanied by any alteration of antioxidant/scavenging enzymatic machinery in the plant roots remains to be studied.

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Herbivore-Induced Volatiles in the Perennial Shrub, *Vaccinium corymbosum*, and Their Role in Inter-branch Signaling

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Abstract Herbivore feeding activates plant defenses at the site of damage as well as systemically. Systemic defenses can be induced internally by signals transported via phloem or xylem, or externally transmitted by volatiles emitted from the damaged tissues. We investigated the role of herbivore-induced plant volatiles (HIPVs) in activating a defense response between branches in blueberry plants. Blueberries are perennial shrubs that grow by initiating adventitious shoots from a basal crown, which produce new lateral branches. This type of growth constrains vascular connections between shoots and branches within plants. While we found that leaves within a branch were highly connected, vascular connectivity was limited between branches within shoots and absent between branches from different shoots. Larval feeding by gypsy moth, exogenous methyl jasmonate, and mechanical damage differentially induced volatile emissions in blueberry plants, and there was a positive correlation between amount of insect damage and volatile emission rates. Herbivore damage did not affect systemic defense induction when we isolated systemic branches from external exposure to HIPVs. Thus, internal signals were not capable of triggering systemic defenses among branches. However, exposure of branches to HIPVs from an adjacent branch decreased larval consumption by 70% compared to those exposed to volatiles from undamaged branches. This reduction in leaf consumption did not result in decreased volatile emissions, indicating that leaves became more responsive to herbivory (or "primed") after being exposed to HIPVs. Chemical profiles of leaves damaged by gypsy moth caterpillars, exposed to HIPVs, or non-damaged controls revealed that HIPV-exposed leaves had greater chemical similarities to damaged leaves than to control leaves. Insect-damaged leaves and young HIPV-exposed leaves had higher amounts of endogenous *cis*-jasmonic acid compared to undamaged and non-exposed leaves, respectively. Our results show that exposure to HIPVs triggered systemic induction of direct defenses against gypsy moth and primed volatile emissions, which can be an indirect defense. Blueberry plants appear to rely on HIPVs as external signals for inter-branch communication.

Keywords Herbivore-induced plant volatiles · External signaling · *Vaccinium corymbosum* · *Lymantria dispar* · Priming

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Introduction

Herbivore feeding often activates direct and indirect defenses in plants, which are mainly regulated by the hormone jasmonic acid (JA; Karban and Baldwin 1997). Direct defenses alter the palatability and/or toxicity of leaf tissues (Agrawal 1998, 1999), while indirect defenses function by attracting natural enemies of herbivores (Turlings et al. 1990;

Vet and Dicke 1992; Dicke and Van Loon 2000). One important type of indirect defense is the emission of a class of volatile organic compounds termed herbivore-induced plant volatiles (HIPVs). Such defense responses against herbivores occur locally, i.e., at the site of feeding, but can also occur systemically, i.e., in non-damaged leaves of damaged plants (Turlings and Tumlinson 1992; Paré and Tumlinson 1998; Mattiacci et al. 2001). Systemic defenses can be induced in plants by internal signals that move primarily through vascular traces (see review by Orians 2005). The movement of internal signals can be restricted by the degree of vascular connectivity between parts of a plant (Orians et al. 2000; Schittko and Baldwin 2003). For instance, leaves with vascular connections to damaged leaves have stronger induction of defenses compared to those without vascular connections (Davis et al. 1991). This restriction can render some systemic regions of an herbivore-damaged plant vulnerable to future herbivory.

In addition to internal signals, plants may respond to external (e.g., airborne) signals emitted from other plants (inter-plant) or from other tissues within a plant (intra-plant). To date, most studies have investigated the role of external cues in inter-plant signaling (Baldwin and Schultz 1983; Dicke et al. 2003). Plants can respond to HIPVs from neighboring plants (Arimura et al. 2002; Farag and Paré 2002; Ruther and Kleier 2005; Ton et al. 2007), although concern exists about whether amounts of airborne signals emitted from damaged plants are abundant enough to maintain concentrations over sufficient distances to trigger the induction of volatiles from neighbors (Preston et al. 2001, 2004; Frost et al. 2008a). As an alternative mechanism, plant volatile signals might not induce defenses immediately but rather sensitize or 'prime' neighboring plants for an enhanced response when attacked by herbivores (reviewed in Frost et al. 2008b). For example, exposure to the green leaf volatiles (*Z*)-3-hexenal, (*Z*)-3-hexen-1-ol, or (*Z*)-3-hexenyl acetate from neighboring plants primes maize plants for defenses (Engelberth et al. 2004). Moreover, HIPVs can induce or prime defenses among systemic parts of the same plant (Frost et al. 2007; Heil and Silva Bueno 2007).

Little is known about the systemic induced responses in long-lived (perennial) shrubs. Individual branches in most shrubs are thought to share limited, if any, vascular connectivity with each other. With such restricted potential for internal systemic wound signaling, shrubs may be particularly vulnerable to mobile herbivores that can move among branches and evade induced defenses. External signals may be able to compensate for such vulnerability. For instance, systemic induced resistance in sagebrush, *Artemisia tridentata* Nutt., is facilitated by air contact with mechanically clipped branches (Karban et al. 2006). Considering that mechanical damage and insect feeding often induce different volatile response in plants (e.g., Mattiacci et al. 1994), the role of HIPVs in mediating systemic induction of resistance in shrubs still remains unclear.

Here, we studied induced local and systemic defense responses in the temperate woody perennial shrub *Vaccinium corymbosum* L. (highbush blueberry; Ericaceae). *V. corymbosum* grows by initiating adventitious shoots from a basal crown, which produce new lateral branches; this type of growth likely constrains vascular connection between shoots and branches within plants, and in turn restricts movement of internal signals. We hypothesized that external signals play an important role in induction of defenses between branches in *V. corymbosum*. This hypothesis was tested by determining whether prior exposure to naturally produced HIPVs from branches damaged by gypsy moth caterpillars activates or primes branches for enhanced defenses against insect attack. We asked the following specific questions: (a) Are volatile responses in *V. corymbosum* induced both locally and systemically by feeding by the generalist herbivore gypsy moth, *Lymatria dispar* L.? (b) Are volatiles induced by methyl jasmonate (MeJA), the volatile derivative of JA, and mechanical damage? (c) Does exposure to gypsy moth-induced volatiles trigger defense responses in neighboring branches? (d) Does exposure to HIPVs reduce larval consumption in neighboring branches? If so, (e) is there an effect on subsequent volatile emissions? We addressed these questions through a series of seven experiments to determine local and systemic emissions of volatiles from blueberry plants and the role of HIPVs in inter-branch signaling. These studies were complemented by three experiments to investigate the degree of vascular connectivity within blueberry plants and to measure internal changes in leaf chemistry.

Methods and Materials

Plants and Insects Actively growing 2-year-old highbush blueberry plants (*V. corymbosum* var. Duke) with 15–20 fully expanded leaves were used in all experiments. 'Duke' highbush blueberry is a vegetatively propagated line (Draper et al. 1987), and therefore genetically identical between individuals. Plants were purchased from A. G. Ammons Nursery (Chatsworth, NJ, USA), grown individually in 1-gal pots (16.5 cm diam) containing a 50:50 mix of sand and peat (Premier Pro-Moss, Red Hill, PA, USA; pH=4–5), watered three times a week, fertilized with 20–20–20 N–P–K all purpose water soluble Miracle-Gro (Scotts Miracle-Gro Products, Marysville, OH, USA), and maintained in a climatic controlled greenhouse (20±2°C; 70±10% RH; 15:9 L/D). All plants used were grown under similar conditions.

To induce defense responses in the blueberry plants, we used gypsy moth (*Lymantria dispar*) caterpillars, which are generalist herbivores that induce direct and indirect defenses in other plant species (Havill and Raffa 1999; Frost et al. 2007; Staudt and Lhoutellier 2007). Gypsy moths are exotic, naturalized pests of oaks and other hardwood species in the northeast USA (Liebhold et al. 1992); they also cause damage to leaves and flowers of blueberry plants, which can be severe in outbreak years. A gypsy moth colony was initiated from egg masses and caterpillars obtained from the USDA-ARS lab, Newark, DE, USA. Field-collected caterpillars were added to the colony prior to the study. Caterpillars were reared on a high wheat germ diet (Bell et al. 1981) and maintained at 24±1°C, 65% RH, and 14:10 h L/D.

Local Induction of HIPVs in Blueberry Leaves

Experiment 1 Blueberry plants were subjected to one of the following treatments: insect damage, mechanical damage, or control. Two branches of each plant were bagged with a spun polyester sleeve (Rockingham Opportunities Corp., Reidsville, NC, USA). All plants, including controls, were bagged to control for any bag effects. In the insect damage treatment, six gypsy moth caterpillars (second to third instars) were placed inside the bags and allowed to feed on plants for 2 days prior to volatile collection. HIPV emissions were collected on day 3. Polyester sleeves remained on plants to prevent insects from escaping. At the end of volatile collection, gypsy moth caterpillars consumed 9.8%±2.1 (SE) per leaf, measured visually as the percent of leaf area removed. In the mechanical damage treatment, damage to plants was inflicted by punching holes to mimic the amount of leaf area removed by gypsy moth. Five leaves per plant were injured with two 7-mm holes located at the base and upper portion of the leaves at the end of days 1 and 2 (1700 h), and volatiles were collected on day 3. The control treatment did not receive insect or mechanical damage. Volatiles from the three treatments were collected concurrently on a given day and each treatment was replicated eight times.

Experiment 2 This experiment investigated the effect of the amount of insect damage on HIPV emissions. Plants were damaged either by zero, two, four, or six gypsy moth caterpillars for 2 days; volatiles were collected on day 3. After volatile collections, larval consumption (in square centimeter) was measured by tracing the area removed from each leaf using Scion Image software (Scion Corporation, Frederick, MD, USA). Treatments were run concurrently and the experiment was replicated three times.

Experiment 3 This experiment tested the role of the jasmonate pathway in the volatile emissions from blueberry leaves. Blueberry plants were treated with 10 mL of either a 1.0- or 1.5-mM solution of MeJA (Sigma-Aldrich, St. Louis, MO, USA) in a 0.1% Tween-20 solution. MeJA was sprayed by using a 2-oz spray bottle (Setco Inc., Cranbury, NJ, USA). Control plants were sprayed with 10 mL of a 0.1% Tween-20 solution. Plants were treated at 1600 h and kept in the greenhouse for 15 h inside a 17-cm diam and 35-cm high Plexiglas chamber (with the entire top and three 2-cm diam holes on the sides of each chamber, covered with a fine mesh for ventilation) prior to volatile collections. After use, Plexiglas chambers were cleaned with tap water and 70% ethanol. Each treatment was replicated four times.

Systemic Induction—Internal Signaling

Experiment 4 One lower branch of a blueberry plant was bagged with a spun polyester sleeve and damaged by placing six (second to third instars) gypsy moth caterpillars inside the bag. The damaged branch remained outside the volatile collection chamber, while the branches above the damaged branch were placed inside the chamber. Caterpillars were allowed to feed on the bottom branch for 2 days. Starting on day 3, volatiles were collected from the undamaged portion of damaged plants. Volatiles were collected for seven consecutive days. Control plants were treated in a similar manner but did not receive insect damage. Treatments were replicated four times.

Vascular Connectivity To determine the degree of vascular connectivity between leaves within a branch and from different branches, we conducted a Rhodamine-B (Sigma-Aldrich) dye assay (Orians et al. 2000). The terminal portion of a lower branch from blueberry plants ($N=7$) was cut so that it could support a floral water pick containing 6 mL solution of the dye (0.25% *w*/*v*). Movement of the dye through the plant was monitored daily for 7 days. After 7 days, the percent of red staining was visually assessed for 30 randomly collected leaves from different positions of the plants. Leaves were sampled from the following positions: branches containing the dye, branches directly above the branch containing the dye (orthostichous branches), branches located across the branch containing the dye, and branches located in a different shoot.

Systemic Induction—External Signaling

Experiment 5 Branches within a plant were either exposed to HIPVs from an adjacent branch or received no exposure to HIPVs. To expose branches to HIPVs, one lower branch was bagged with a polyester sleeve and six (second to third instars) gypsy moth caterpillars were placed inside bags. This treatment allowed movement of significant amounts of

volatiles outside the bags as indicted by experiments testing local induction of volatiles (see "Results"). The aerial portion of the plant was caged inside a Plexiglas chamber similar to those described above. Insects were allowed to feed on plants for 2 days, such that adjacent branches were exposed to volatiles emitted from the induced branch. After exposure (day 3), exposed branches were placed inside the volatile collection chamber, leaving the insect-injured branch outside. Volatiles from HIPV-exposed branches were collected, and the amount of leaf area consumed from the lower branch was measured. Each treatment was replicated four times.

Experiment 6 To determine whether plants were "primed" after HIPV exposure, eight plants were treated as in experiment 5. In half of the plants, branches were exposed to HIPVs from an induced adjacent branch, while the other half was exposed to undamaged branches. On day 3, four early second instar gypsy moth caterpillars were placed on each plant, and volatiles were collected. The size of caterpillars was standardized by obtaining initial larval weights; each set of four caterpillars averaged 50±5 mg. After volatile collections, leaves initially damaged by gypsy moth caterpillars, as well as those that were damaged on day 3, were excised, and the amounts of leaf area consumed were measured. Treatments were replicated four times.

Experiment 7 An additional experiment similar to experiment 6 was conducted but 25×25 cm plastic ziplog bags (National Bag, Hudson, OH, USA) were used instead of polyester sleeve bags. Ziplog bags prevented HIPV contact with leaves from undamaged branches within a plant. Each treatment was replicated four times.

Chemical Analyses

HIPV Collection and Analysis HIPV emissions were sampled in the greenhouse (20±5°C; 70±10% RH; 15:9 h L/D). Volatiles were collected using a push–pull system (Rodriguez-Saona et al. 2001, 2006). The above ground portion of potted plants (stem, branches, and leaves), including caterpillars in the insect damage treatment, were placed inside a 4-L volatile collection chamber (Analytical Research Systems, Inc. Gainesville, FL, USA). A two-piece guillotine supported the base of plants. Purified air entered the top of each chamber at a rate of 2 L/min. Volatiles were collected in traps filled with 30 mg of Alltech Super-Q adsorbent (Analytical Research Systems, Inc), by pulling air from the chambers at a rate of 1 L/min. The volatile collection apparatus consisted of four chambers, allowing for simultaneous collections of volatiles from four different plants. Collections were conducted during daytime from 0900 to 1700 h. After collection, all leaves from plants were harvested, oven dried at 60°C, and weighed, and chambers were rinsed with tap water and 70% ethanol.

The collected volatiles from Super-Q traps were eluted with dichloromethane (150 μL), and 400 ng of *n*-octane (Sigma-Aldrich) were added as internal standard. Separation and quantification of compounds was done on a Hewlett Packard 6890 Series Gas Chromatograph (GC), equipped with a flame ionization detector (FID) and an Agilent HP-1 column (10 m×0.53 mm×2.65 μm), and using He as carrier gas (constant flow=5 mL/min, velocity=39 cm/s). The temperature program started at 40°C kept for 1 min, increased at 14°C/min to 180°C (2 min), then at 40°C/min to 200°C, and kept at 200°C for 2 min. Individual compounds (nanogram per gram of dry material per hour) were quantified based on comparison of peak areas from GC–FID with that of the internal standard.

Identification of compounds was carried out on a Varian 3400 GC coupled to a Finnigan MAT 8230 mass spectrometer (MS), equipped with a Supelco MDN-5S column (30 m×0.32 mm×0.25 μm), and with He as the carrier gas. The program started at 35°C (1 min), increased at 4°C/min to 170°C, then at 15°C/min to 280°C. The MS data were acquired and processed in a Finnigan MAT SS300 data system. Compounds were identified by comparison of spectral data with those from NIST library and by GC retention index (Jennings and Shibamoto 1980; Adams 2001) and confirmed by comparing their retention times with those of commercially available compounds.

A completely randomized one-way ANOVA (Minitab Statistical Software, Release 13, State College, PA, USA) was used to determine differences in volatile emissions among treatments. If a treatment effect was significant, a Tukey test was conducted. Differences in volatile emissions and leaf area eaten between two treatments were determined using *t* tests. Data were transformed by $\ln(x)$, or $\ln(x+0.05)$ if data included zero values, prior to analyses. The amount of insect damage was correlated against the amount of volatiles emitted.

Quantification of Jasmonic Acid, Salicylic Acid, and Linolenic Acid In a separate experiment, a lower branch of three blueberry plants was damaged by six (second to third instars) gypsy moths, as described in experiment 5. On day 3, all leaves from each plant were collected for phytohormone analyses. Leaves from the insect-damaged branch and from young and old leaves of HIPV-exposed branches were sampled separately (young leaves were those soft leaves in branches at the top of plants, and old leaves were those hard leaves in branches at the base of the plant). Samples were also collected from three other plants that received no insect damage (controls). Samples were placed

directly into liquid N_2, and care was taken to not allow the tissue to thaw at any point prior to extraction.

Extraction and quantification were performed as previously described (Schmelz et al. 2003, 2004). Briefly, ~100 mg frozen ground tissue of each sample was transferred to 2-mL screw-cap FastPrep tubes (Qbiogene, Carlsbad, CA, USA) containing ~1 g of Zirmil beads (1.1 mm; SEPR Ceramic Beads and Powders, Mountainside, NJ, USA). Dihydro-JA and $^2H_6$-SA (CDN Isotopes, Pointe-Claire, Quebec, Canada) and gamma-LA (Matreya LLC, Pleasant Gap, PA, USA) were added as internal standards to the 2-mL tubes before sample addition (100 ng each). The samples were mixed with 400 mL of 1-propanol/H_2O/HCl (2:1:0.002) and shaken for 40 s in a FastPrep FP 120 tissue homogenizer (Qbiogene). Dichloromethane (1 mL) was added to each sample, re-shaken for 40 s in the homogenizer, and centrifuged at 10,000×*g* for 1 min. The bottom organic phase (dichloromethane) was transferred to a 4-mL glass screw-cap vial and evaporated by a constant air stream (~10 min). Each sample was reconstituted in 100 mL diethyl ether/methanol (9:1, *v*/*v*), and carboxylic acids were converted into methyl esters by adding 2.3 μL of a 2.0-M solution of trimethylsilyldiazomethane in hexane (Sigma-Aldrich). The vials were capped, vortexed, and incubated at room temperature for 30 min. The reaction was stopped by the addition of hexane/acetic acid (78:12 *v*/*v*), followed by brief vortexing and incubation for 30 min at room temperature. Volatile metabolites were separated from the complex mixture by vapor-phase extraction as described in Schmelz et al. (2004). The methylated products were eluted with 150 μL of dichloromethane and analyzed by chemical ionization−GC–MS.

Differences in amounts of JA, salicylic acid (SA), and linolenic acid (LA) between damaged and undamaged leaves were determined by using *t* tests. Differences in the amounts of these phytohormones between HIPV-exposed and -unexposed leaves were analyzed with a two-way ANOVA with treatment (exposed vs. unexposed) and leaf age (young vs. old) as main effects. If significant differences were found, a Tukey test was conducted. Data were transformed by ln(*x*) prior to analysis.

Fourier-Transform Infrared Phenolic extracts of each treatment were compared by using Fourier-transform infrared (FT-IR) to investigate if differences exist in the chemical profiles. This technique allowed us to compare spectral differences among treatments across broad wavenumbers (4,000–600 cm^{-1}), particularly those associated with phenolic compounds (~1,400 to 1,000 cm^{-1}). Branches of 12 different blueberry plants were either (a) exposed to HIPV emissions, (b) damaged locally by six gypsy moth caterpillars, or (c) left undamaged, as described above. Leaves from each branch were excised after 2 days of treatment, frozen in liquid N_2, stored at −70°C, and used for FT-IR analysis. Frozen leaves were sonicated (sonic dismemberator model 100; Fisher Scientific, Pittsburgh, PA, USA) with 70% aqueous acetone (*v*/*v*) for 30 s with an output of 8 W. Chloroform was added to the suspension, and phase separation was achieved by using a microcentrifuge (Model 5415R, Eppendorf, Westbury, NY, USA) operating at 16,100×*g* for 3 min. The top aqueous layer was collected, concentrated under a stream of nitrogen, and further fractionated by using a modified solid-phase extraction (SPE) method described by Giusti et al. (1999). Briefly, the sample was loaded onto a Sep-Pak Vac (1 g) C18 cartridge (Waters, Milford, MA, USA) that had been preconditioned with 10 mL of acidified methanol (0.1% HCl) and 10 mL of acidified DD water (0.1% HCl) and then washed with 5 mL of acidified DD water. Phenolics were eluted from the column with 5 mL of acidified methanol. An aliquot of the phenol-rich fraction was dried under nitrogen flow and reconstituted by adding 500 μL of 30% methanol containing 0.1% HCl. An aliquot (10 μL) was applied onto a SpectCONC-IR (Tienta Sciences, Indianapolis, IN, USA) slide and vacuum-dried to yield a homogeneous film. The use of the SpectCONC-IR slide provides a high IR reflectance surface and the ability to concentrate samples up to ten times due to the presence of a hydrophobic coating.

Infrared spectra were collected on an infinity corrected FT-IR microscope (UMA 600 series IR microscope interfaced with a FTS Excalibur 3500GX FTIR spectrometer; Varian, Walnut Creek, CA, USA), equipped with a motorized x–y stage and a broadband mercury cadmium telluride detector. The spectrometer was controlled by Win-IR Pro control software programmed to collect spectra over the frequency range 4,000–600 cm^{-1}. The spectral resolution was 4 cm^{-1} and to improve the signal-to-noise ratio, 128 spectra were co-added and averaged. Spectra were displayed in terms of absorbance as calculated from the transmittance spectra by using the Win IR Pro software. Infrared spectral data transformations included area normalization and second derivative based on a Savitzky–Golay polynomial filter. Sample residual and Mahalanobis distance were used for outlier diagnostics.

Spectral differences among treatments were determined by using multivariate data analyses. The spectra were imported as JCAMP-DX files into the Pirouette statistical software (Version 3.02 for Windows NT, Infometrix, Inc., Woodinville, WA, USA), and multivariate training models were produced using soft independent modeling of class analogy (SIMCA). Analysis by SIMCA, a supervised method for sample classification, consists of assigning training sets to classes and then a principal component model is created for each class with different confidence

regions (De Maesschalck et al. 1999). Class projection plots were used to illustrate the ability of SIMCA to differentiate FT-IR data based on the first three principal components. This plot was used for the visualization of clustering among samples (sample patterns, groupings, or outliers). SIMCA analysis was carried out by Pirouette pattern recognition software (Version 3.02 for Windows NT, Infometrix, Inc., Woodinville, WA, USA). The interclass distances were used as a diagnostic for a SIMCA model, which essentially is the Euclidian distance between the centers of each group where large interclass distances imply well-separated classes. SIMCA's discrimination power was used as a measure of variable importance (i.e., infrared frequency), which contributes to the development of the classification models (Dunn and Wold 1995).

Results

Local Induction of HIPVs in Blueberry Leaves

Twenty-two volatile compounds were identified by GC–MS from blueberry leaves (Fig. 1; Table 1). Mechanical damage and feeding by gypsy moth caterpillars increased volatiles emissions from blueberries by four- and ninefold, respectively, compared to controls (Table 1). Mechanical damage increased emissions of (*Z*)-3-hexenyl acetate, benzene acetonitrile, γ-cadinene, α-farnesene, and caryophyllene oxide ($P \leq 0.05$). In addition to these, feeding by gypsy moth increased volatile emissions of 12 other, mostly terpene, compounds including α-pinene, eucalyptol, linalool, myrcenone, caryophyllene, humulene, and β-

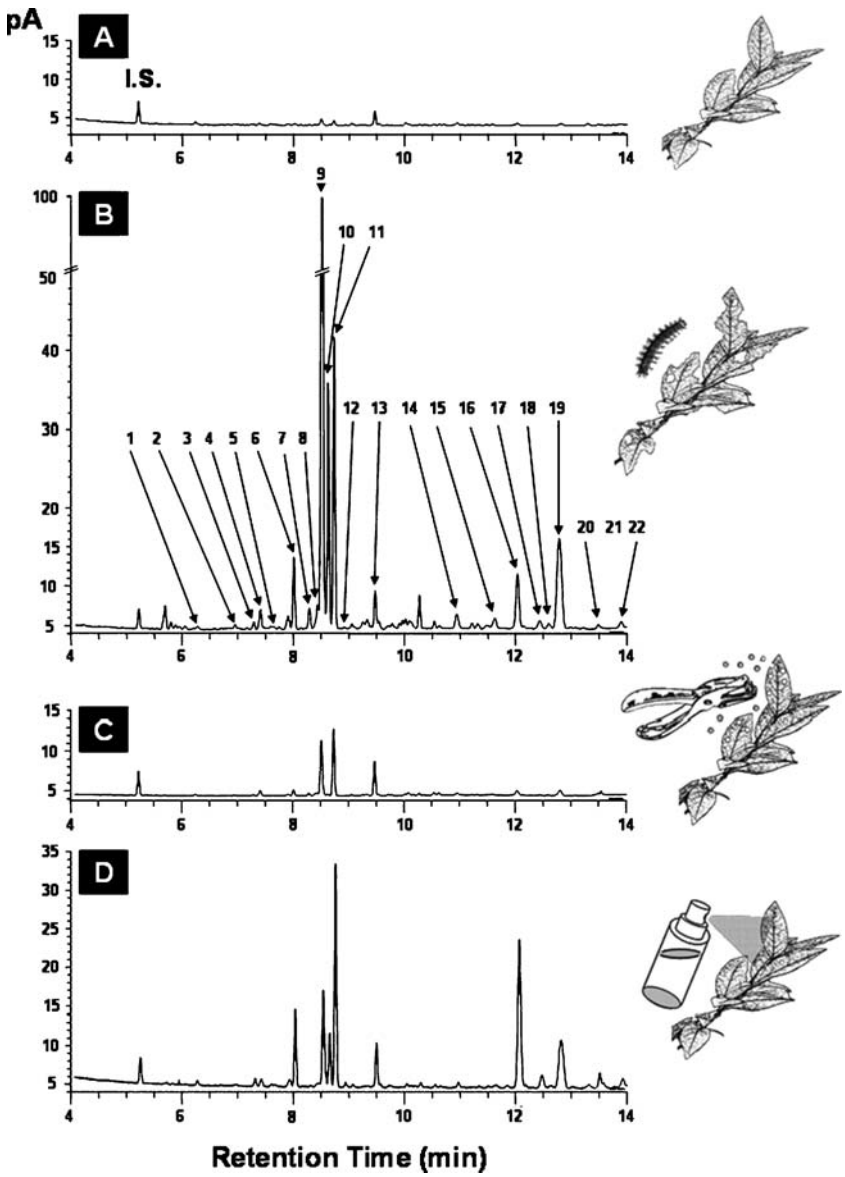

Fig. 1 Representative GC–FID profiles of headspace volatiles collected from 2-year-old highbush blueberry, *Vaccinium corymbosum*, plants. Treatments are: undamaged plants (**A**), plants damaged by six, second to third instar, gypsy moth caterpillars (**B**), plants damaged mechanically (**C**), and plants treated with 1.0 mM MeJA (**D**). Compound names **1**–**22** are listed in Tables 1 and 2. *I.S.* internal standard (*n*-octane). Volatiles were collected from 0900 until 1700 h

Table 1 Effects of mechanical and gypsy moth damage on volatile emissions from highbush blueberries, *V. corymbosum*

Compound name[a]	Control[b]	Mechanical damage	Gypsy moth damage
1. 2,5 Hexanedione	*10.49±5.61 a*	*6.41±2.42 a*	*33.88±6.21 b*
2. α-Pinene	*3.23±2.14 a*	*1.02±1.02 a*	*10.43±2.82 b*
3. β-Pinene	7.72±6.06 a	48.18±26.08 a	8.07±3.55 a
4. cis-3-hexenyl acetate	*19.70±6.18 a*	*66.02±19.56 b*	*76.11±17.49 b*
5. Hexyl acetate	15.36±6.11 a	19.19±8.14 a	39.13±6.37 a
6. Eucalyptol	*30.33±15.24 a*	*126.54±56.00 ab*	*422.32±183.60 b*
7. Linalool oxide cis	*7.36±1.84 a*	*18.20±5.14 ab*	*76.10±21.45 b*
8. Linalool oxide trans	*6.49±2.22 a*	*22.78±4.66 ab*	*75.98±23.54 b*
9. Linalool	*77.37±23.24 a*	*434.43±144.94 ab*	*1,589.76±567.58 b*
10. Myrcenone	*21.53±11.20 a*	*45.20±17.01 a*	*1,240.85±719.11 b*
11. Benzene acetonitrile	*11.11±3.57 a*	*444.63±157.81 b*	*1,131.21±481.86 b*
12. Phenylethyl acetate	*15.01±6.46 a*	*12.21±4.85 a*	*40.26±5.69 b*
13. Methyl salicylate	46.21±12.03 a	67.50±25.01 a	123.01±32.31 a
14. β-Bourbonene	*43.95±24.45 ab*	*21.31±7.45 a*	*93.38±26.19 b*
15. Copaene	21.43±8.14 a	19.45±7.64 a	81.75±32.66 a
16. Caryophyllene	*8.85±6.44 a*	*78.77±28.50 ab*	*814.67±418.35 b*
17. Humulene	*0.00±0.00 a*	*8.05±4.15 a*	*145.69±85.00 b*
18. β-Farnesene	*0.33±0.33 a*	*3.40±2.77 a*	*28.37±6.21 b*
19. γ-Cadinene	*21.87±7.38 a*	*61.30±15.65 b*	*1,005.97±278.92 c*
20. α-Farnesene	*5.06±2.77 a*	*39.28±14.50 b*	*62.83±24.53 b*
21. Farnesol	2.01±2.01 a	2.59±2.59 a	14.26±6.90 a
22. Caryophyllene oxide	*7.58±5.28 a*	*17.10±5.81 b*	*85.83±30.59 b*
Totals	382.98±100.23 a	1,562.28±509.36 b	3,439.49±1,140.69 c

Italicized data indicate differences among treatments (ANOVA, $P<0.05$). Same letters within rows are not significantly different ($P>0.05$)
[a] Compound identities were confirmed by GC–MS
[b] Values are in nanogram per gram per hour ±SE, $N=8$

farnesene ($P\leq0.05$; Table 1). There was a strong, positive correlation between the amount of gypsy moth damage and volatile emissions ($F=41.4$; $df=1, 10$; $P<0.001$; $r^2=0.79$; Fig. 2).

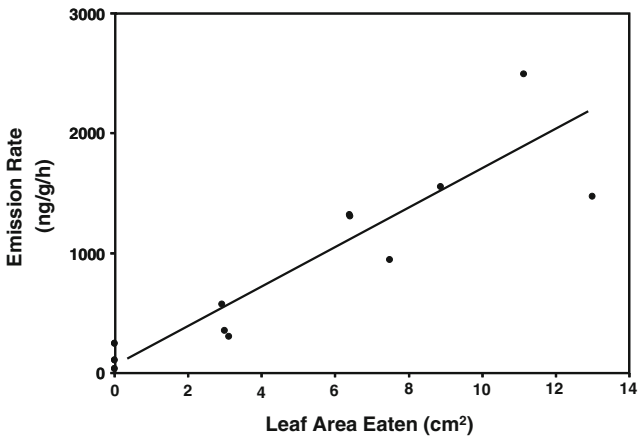

Fig. 2 Correlation of the volatile emission rates with the amount of leaf area eaten by gypsy moth caterpillars in blueberry plants. Emission rates were calculated by dividing the total relative amounts of volatiles by the dry weight of plant material and the total number of hours for each collection. The relative amount of volatiles for a particular compound was calculated as the ratio of its peak area to the peak area of the internal standard. Amount of leaf area eaten was measured by tracing the area removed from each leaf by caterpillars

Compared to caterpillar feeding, the MeJA treatment induced 11 out of the 17 compounds induced by gypsy moth (Table 2). Compounds significantly increased by gypsy moth but not by MeJA were 2,5 hexanedione, α-pinene, (*Z*)-3-hexenyl acetate, *cis*-linalool oxide, β-bourbonene, and caryophyllene oxide. Five chemicals (β-pinene, hexyl acetate, methyl salicylate, copaene, and farnesol) were not increased by any treatment.

Systemic Induction—Internal Signaling

There was no evident induction of volatiles from undamaged leaves of gypsy moth-damaged plants 7 days after initial feeding damage (day 3: control (undamaged)= 167.7±54.4 ng/h (mean ± SE volatile emissions), treatment (systemic induction)=173.8±63.3 ng/h, $t=0.15$, $P=0.88$; day 4: control=126.9±35.5 ng/h, treatment=140.4±41.7 ng/g, $t=0.11$, $P=0.92$; day 5: control=129.6±59.7 ng/h, treatment=121.5±52.4 ng/h, $t=0.07$, $P=0.95$; day 6: control=171.2±96.7, treatment=88.8±28.0, $t=0.11$, $P=0.92$; day 7: control=181.6±41.0, treatment=230.8±92.5, $t=0.08$, $P=0.94$).

Vascular connectivity among leaves depended heavily on their relative position. On days 1–3, we observed very slow movement of the red dye such that there was little staining

Table 2 Effects of exogenous applications of methyl jasmonate on volatiles emissions from highbush blueberries, *V. corymbosum*

Compound name	Control[a]	1.0 mM	1.5 mM
1. 2,5 Hexanedione	9.16±1.83 a	10.04±2.57 a	5.74±0.99 a
2. α-Pinene	0.00±0.00 a	2.20±2.20 a	2.19±1.32 a
3. β-Pinene	15.10±10.58 a	23.07±13.77 a	28.69±4.48 a
4. *cis*-3-hexenyl acetate	11.37±4.77 a	17.87±6.24 a	23.48±3.72 a
5. Hexyl acetate	4.18±1.50 a	3.82±2.57 a	2.09±2.09 a
6. Eucalyptol	*26.77±14.89 a*	*77.30±25.86 ab*	*222.27±27.87 b*
7. Linalool oxide cis	1.50±1.50 a	9.42±5.62 a	8.81±2.16 a
8. Linalool oxide trans	*3.01±1.86 a*	*9.21±1.59 ab*	*17.99±5.22 b*
9. Linalool	*28.74±7.53 a*	*130.60±28.62 b*	*524.43±117.20 c*
10. Myrcenone	*8.21±4.03 a*	*40.96±12.98 ab*	*98.71±35.80 b*
11. Benzene acetonitrile	*15.00±8.05 a*	*171.94±65.00 b*	*418.68±101.35 b*
12. Phenylethyl acetate	*0.00±8.63 a*	*5.00±9.55 b*	*11.04±11.41 c*
13. Methyl salicylate	35.15±0.00 a	58.11±5.00 a	65.23±2.70 a
14. β-Bourbonene	15.48±5.27 a	12.79±2.70 a	19.07±4.82 a
15. Copaene	5.23±1.76 a	17.28±10.27 a	18.89±3.61 a
16. Caryophyllene	*38.57±28.81 a*	*387.13±195.91 b*	*961.88±191.20 b*
17. Humulene	*2.78±2.78 a*	*10.61±7.23 b*	*117.96±21.66 c*
18. β-Farnesene	*0.00±0.00 a*	*27.42±21.87 b*	*16.59±2.89 b*
19. γ-Cadinene	*8.52±3.23 a*	*118.30±25.11 b*	*406.67±106.40 b*
20. α-Farnesene	*2.60±2.60 a*	*25.61±7.01 b*	*82.66±20.30 b*
21. Farnesol	0.00±0.00 a	3.81±2.55 a	9.61±5.94 a
22. Caryophyllene oxide	14.06±9.52 a	47.09±19.56 a	82.34±21.65 a
Totals	245.42±108.03 a	1,209.59±376.28 b	3,145.04±435.84 c

Italicized data indicate differences among treatments (ANOVA, $P<0.05$). Same letters within rows are not significantly different ($P>0.05$)
[a] Values are nanogram per gram per hour ±SE, $N=4$

in leaves other than those in branches containing the dye. On day 4, some staining occurred in branches close to those containing the dye (mainly in orthostichous branches, i.e., vertically aligned in a shoot), but most staining was limited to one or two leaves close to the shoot. After 1 week, we found high vascular connectivity between leaves within a branch (Fig. 3A), intermediate-to-low connectivity between leaves from orthostichous branches (Fig. 3B), low connectivity between leaves from non-orthostichous branches (i.e., on opposite sides of a shoot; Fig. 3C), and a lack of connectivity between leaves from branches located on different shoots (Fig. 3D).

Systemic Induction—External Signaling

Herbivore-induced plant volatiles mediated systemic resistance among blueberry branches. There was no difference between volatile concentrations emitted from branches exposed to HIPVs vs. those not exposed to HIPVs ($t=0.58$; $P=0.59$; Fig. 4A). However, *L. dispar* caterpillars fed on leaves previously exposed to HIPVs consumed 70% less leaf material than did those fed on unexposed control leaves ($t=8.62$; $P=0.003$; Fig. 4B). Despite the reduction in leaf consumption, total volatile emissions between HIPV-exposed and HIPV-unexposed branches were similar (total volatile emissions for HIPV-unexposed branches=1,127.4±180.9 ng/g/h, HPIV-exposed branches=1,313.9±435.1 ng/g/h; $t=0.01$; $P=0.99$). As a result, amounts of volatiles emitted per amount of leaf area consumed in HIPV-exposed branches were fourfold higher compared to unexposed

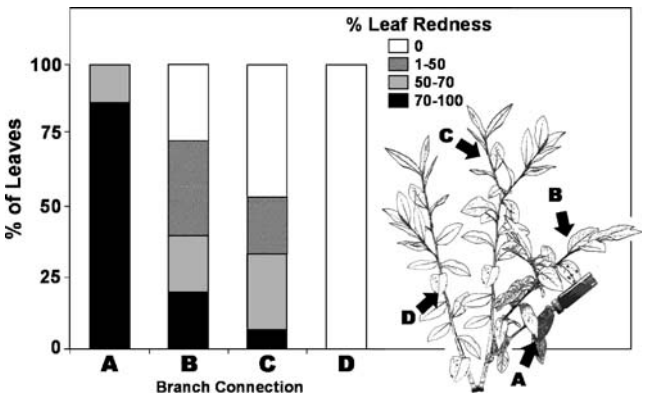

Fig. 3 Rhodamine-B (red dye) was used to determine the degree of vascular connectivity between leaves within a branch, and from different branches within blueberry plants. (*Left*) Percent of leaves with different amounts of red staining. The amount of red staining (0%, 1–50%, 50–70%, and 70–100%) was visually assessed for 30 randomly collected leaves from each of four positions *A–D* of blueberry plants ($N=7$). Leaf samples were taken after 7 days. (*Right*) Location of leaves sampled within a plant. Leaves were sampled from: branches containing the dye (*A*), branches directly above the branch containing the dye (*B*), branches located across the branch containing the dye (*C*), and branches located in a different shoot (*D*)

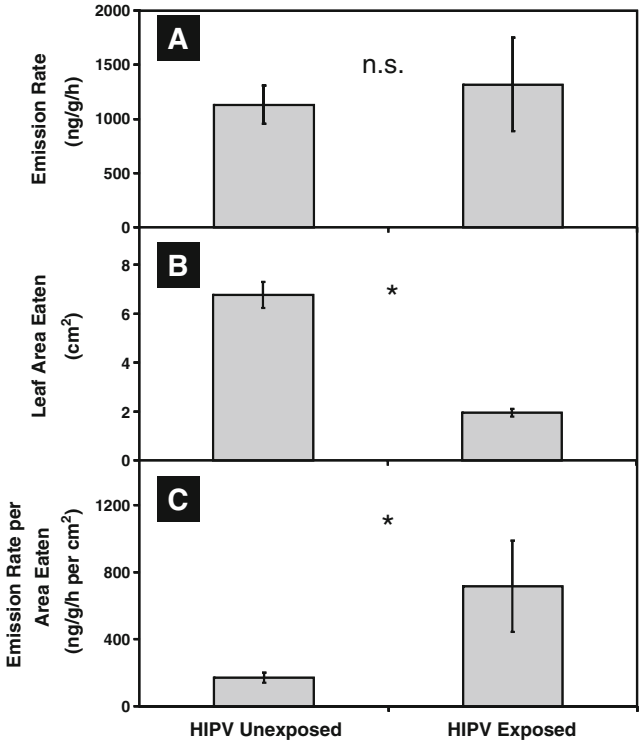

Fig. 4 Induction and priming of blueberry volatiles by herbivore-induced plant volatiles (HIPVs). **A**) Volatile emission rates of blueberry branches that were either exposed to HIPVs (*HIPV Exposed*) from an adjacent branch of the same plant, or received no exposure to HIPVs (*HIPV Unexposed*). **B**) Amount of leaf area eaten by gypsy moth caterpillars on HIPV-unexposed and HIPV-exposed branches. **C**) Amount of volatiles emitted per amount of leaf area consumed in HIPV-unexposed and HIPV-exposed branches. *Asterisks*, significant differences between treatments (N=4, $P \leq 0.05$); *n.s.* not significant

branches (t=2.78; P=0.05; Fig. 4C), indicating that leaves from HIPV-exposed branches were more responsive to herbivory (i.e., they were primed).

The priming effect was eliminated when undamaged branches were prevented from being exposed to HIPVs from damaged branches within a plant (emission rate per leaf area eaten=588.7±159.4 ng/g/h per cm^2 for HIPV-unexposed branches and 399.4±120.5 ng/g/h per cm^2 for HIPV-exposed branches; t=0.97; P=0.376). Differences in priming between these experiments were not due to differences in the amounts of leaf area consumed by gypsy moth in the lower branches of plants (initial damage; total amount of leaf area consumed=7.8±4.6 cm^2 (experiment 6) and 12.4±3.2 cm^2 (experiment 7); t=1.64, P=0.16).

Internal Leaf Chemistry

Endogenous JA was induced by both gypsy moth feeding and HIPV exposure. Gypsy moth feeding strongly increased amounts of JA at the site of damage (*cis*-JA: control=0.9±0.2 ng/g, treatment=94.6±34.4 ng/g, t=10.44, P=0.002; *trans*-JA: control=1.7±0.5 ng/g, treatment=27.8±8.2, t=6.17, P=0.009). More surprisingly, leaf exposure to HIPVs had an effect on amounts of *cis*-JA concentrations (F=6.73; df=1, 8; P=0.03). This effect was influenced by leaf age, such that *cis*-JA levels in young leaves exposed to HIPVs were 20 times greater than unexposed leaves (significant treatment-by-age interaction; F=5.33; df=1, 8; P=0.05; Fig. 5). Older leaves did not have higher concentrations of *cis*-JA, and exposure to HIPVs had no effects on amounts of *trans*-JA (F=0.09; df=1, 8; P=0.77).

In contrast to JA concentrations, no differences in the concentrations of SA and LA were found between control and damaged leaves (SA: control=3.0±1.6 ng/g, treatment=5.8±1.5 ng/g, t=1.4, P=0.256; LA: control=235.6±131.3 ng/g, treatment=93.9±43.6 ng/g, t=0.90, P=0.434). Moreover, exposure to HIPVs had no effect on concentrations of SA (F=2.41; df=1, 8; P=0.16) or LA (F=1.77; df=1, 8; P=0.22). However, SA concentrations were higher in younger leaves compared to old leaves within a plant (F=22.7; df=1,

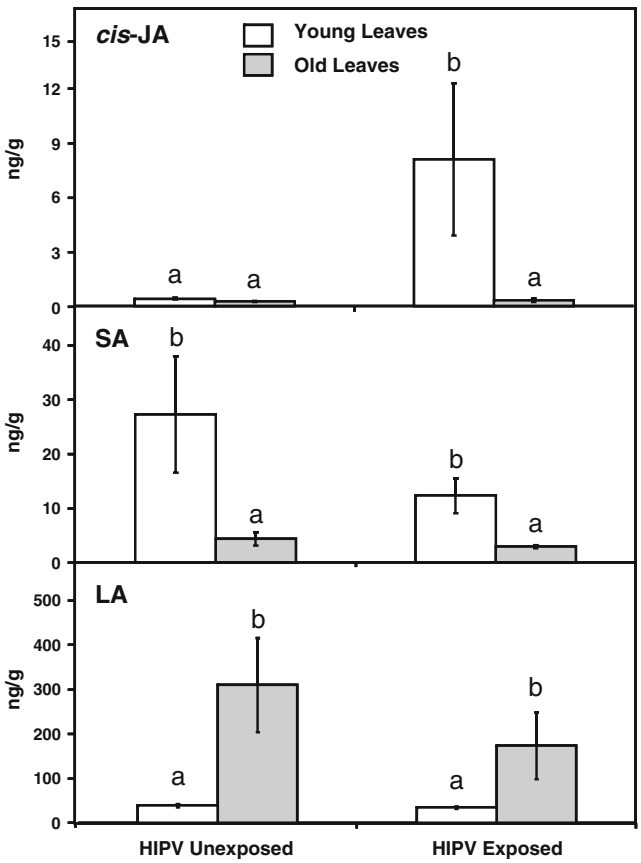

Fig. 5 Levels of endogenous jasmonic acid (*cis-JA*), salicylic acid (*SA*), and linolenic acid (*LA*) in blueberry leaves from branches that were either exposed to HIPVs from adjacent branches within a plant (*HIPV Exposed*), or not exposed (*HIPV Unexposed*). Young and old leaves were analyzed separately. *Different letters above bars* indicate differences in the levels between treatments (N=3, $P \leq 0.05$)

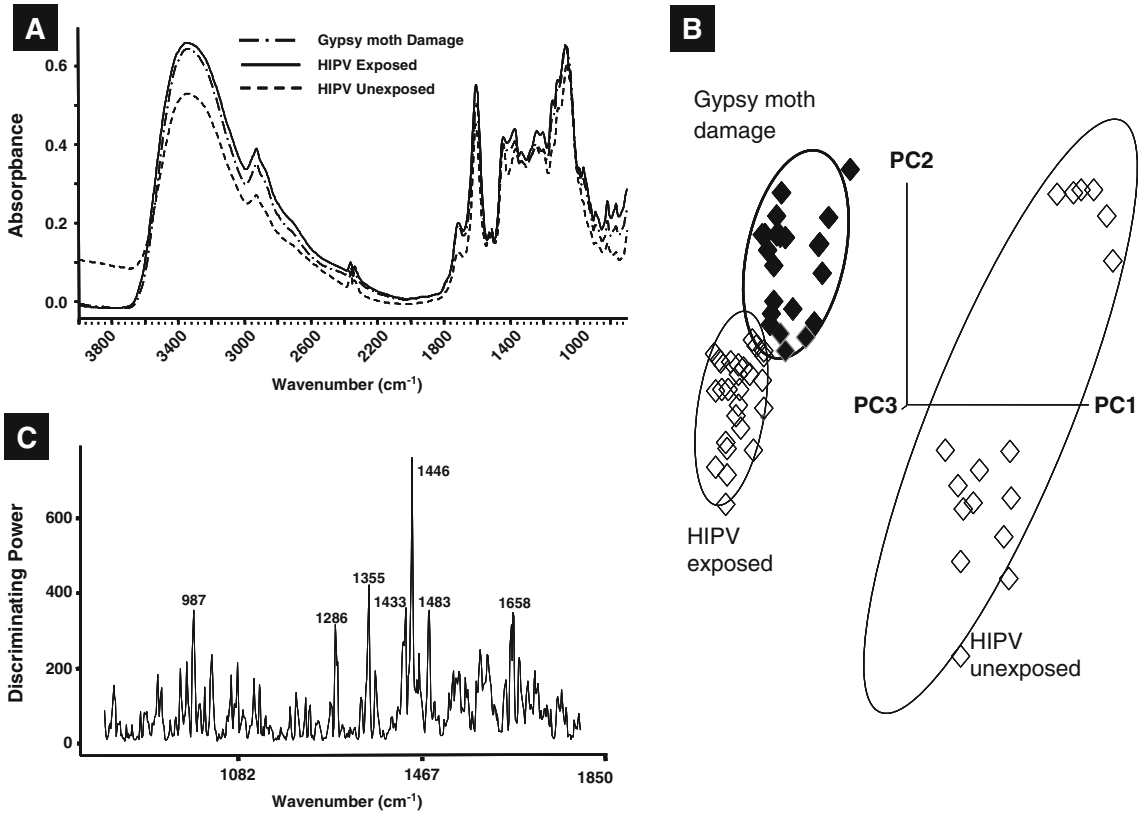

Fig. 6 **A**) Characteristic FT-IR spectrum from blueberry leaves that were either damaged by the gypsy moth, exposed to HIPVs (*HIPV Exposed*), or not exposed to HIPVs (*HIPV Unexposed*). **B**) A three principal component analysis showing good class separation among treatments. **C**) Soft independent modeling of class analogy (SIMCA) discrimination power showing bands associated with the classification of the samples; these bands are related to vibrations of aromatic and/or glycosylated functional groups such as quinone (1,670 cm^{-1}), the benzene structure (1,584 and 1,450 cm^{-1}), secondary C–O–H (1,317 and 1,171 cm^{-1}) structure of phenolic –OH groups and sugar moieties, and the pyranose rings (962 cm^{-1})

8; P=0.001); while LA concentrations in young leaves were lower than in old leaves (F=34.04; df=1, 8; P<0.001; Fig. 5). There was no effect of leaf age on amounts of *trans*-JA or treatment-by-age interaction for *trans*-JA, SA, and LA (all P values>0.05).

Infrared microspectroscopy (FT-IR) detected differences in chemical profiles among gypsy moth damaged, HIPV-exposed, and HIPV-unexposed blueberry leaves. FT-IR further indicated that the HIPV-exposed leaves were more chemically similar to leaves damaged by gypsy moths than to control leaves. Observation of the spectra showed differences among treatments particularly in the frequency region ranging from 1,800 to 900 cm^{-1} (Fig. 6A). The SIMCA pattern recognition model showed good class separation among treatments, zero misclassifications, "tight" cluster of samples, and large distances (2.01 to 8.04) between classes (Fig. 6B). SIMCA models showed interclass distance values of 6.5 for HIPV-unexposed vs. HIPV-exposed leaves, and 8.0 for HIPV-unexposed vs. gypsy moth damage leaves; class distances above 3.0 are generally considered good for discrimination (Kvalheim and Karstang 1992). Interestingly, the interclass distance between clusters for spectra collected from the phenolic fraction of HIPV-treated and gypsy moth damage leaves was 2.01, indicating similarities in their chemical profiles. Using SIMCA's discrimination power, we found bands associated with the classification of the samples that are related to vibrations of aromatic and/or glycosylated functional groups such as quinone (1,670 cm^{-1}), the benzene structure (1,584 and 1,450 cm^{-1}), secondary C–O–H (1,317 and 1,171 cm^{-1}) structure of phenolic −OH groups and sugar moieties, and the pyranose rings (962 cm^{-1}; He et al. 2007; Fig. 6C).

Discussion

This study provides the first insights into the volatile response of the perennial shrub *V. corymbosum* to herbivory by gypsy moth caterpillars and new evidence for the role of HIPVs in intra-plant signaling. As expected, caterpillar feeding increased endogenous JA concentrations and volatile emission rates at the site of damage. In addition, exogenous MeJA application induced 65% of the same

volatiles induced by caterpillar feeding, indicating that the JA pathway plays a key role in the induction of HIPVs in *V. corymbosum*. The JA pathway has been shown to induce the emissions of volatiles in several other plant species (e.g., Hopke et al. 1994; Boland et al. 1995; Gols et al. 1999; Rodriguez-Saona et al. 2001). Under control conditions (i.e., no previous exposure to volatiles), HIPV emission rates correlated linearly with the amounts of damage and exogenous MeJA application within the range tested in this study, such that greater damage and MeJA concentration triggered higher volatile emissions. However, undamaged leaves with previous exposure to naturally occurring HIPVs had higher volatile emission rates than were expected once they were fed upon by gypsy moth, which indicates that HIPV exposure had a priming effect on undamaged leaves. Since gypsy moth caterpillars and their excrement are odorless (Staudt and Lhoutellier 2007), they are not a confounding source of volatiles in our experiments.

Based on our results, *V. corymbosum* cannot and does not rely on internal signals to induce defenses among vascularly isolated branches. Blueberry plants have a complex architectural structure that limits vascular connectivity among branches. As a result, systemic responses to herbivores are highly variable in space and time. For example, when we collected volatiles from the upper branches of damaged plants, we failed to find a systemic volatile response 7 days after initial insect damage. When observed in other systems, systemic responses in plants often occur within 7 days after damage (Mattiacci et al. 2001). From an ecological perspective, systemic responses that fail to activate within 7 days are likely to fail to provide adequate defenses. While priming (rather than induction) of systemic volatile emissions via vascular connections has been shown in *Populus* saplings (Frost et al. 2007), the important point for blueberry plants is that vascular connections are limited.

In contrast to vascular systemic signaling, airborne compounds are capable of acting as systemic wound signals among branches of *V. corymbosum*. Indeed, our results indicate that HIPV-mediated signaling is an effective mediator of systemic defenses and, more importantly, resistance against herbivory. For instance, we found that exposure to HIPVs reduced gypsy moth feeding by 70% in adjacent leaves from different branches compared to controls. Blocking exposure to HIPVs prevented these results, indicating that HIPVs are necessary for intra-plant signaling among vascularly isolated branches. Previous studies also have shown that exposure of HIPVs induces resistance against insects between (Karban et al. 2003; Ton et al. 2007) and within (Heil and Silva Bueno 2007) plants. Although the exact identity of compounds responsible for the changes in chemical profiles among treatments remains unknown, they are likely associated with polyphenolic compounds, and thus, it is possible that increases in *cis*-JA in HIPV-exposed leaves induced production of antiherbivore compounds. Alternatively, HIPV exposure might not trigger insect resistance per se, but rather prime exposed leaves for an increased response to insect damage. Although we found significantly higher levels of *cis*-JA in exposed leaves, insect-damaged leaves had more than ten times higher levels of *cis*-JA than did HIPV-exposed leaves. HIPV-exposed leaves had chemical profiles that were more similar, but not identical, to damaged leaves than to controls (Fig. 6). Thus, the levels of JA in HIPV-exposed leaves may not have been sufficient to reduce feeding by gypsy moth, but rather may have primed these leaves to respond more rapidly and/or strongly to insect attack. For instance, corn seedlings previously exposed to HIPVs from neighboring plants induce rapid production of JA in response to a wounding treatment (Engelberth et al. 2004).

The effects of HIPVs on *cis*-JA induction were greater in young leaves than older leaves within a plant. This agrees with previous results that showed volatile-mediated systemic induced resistance only in young, actively growing sagebrush (Shiojiri and Karban 2006). Gypsy moth occurrence in *V. corymbosum* synchronizes with the plant's ontogeny such that caterpillars become active early in the season when blueberry plants are developing new growth. Gypsy moth caterpillars have a preference for young *V. corymbosum* leaves (C.R.-S. personal observation), thus protection of young leaves might be adaptive.

Although HIPV exposure did not induce volatile emissions, HIPV-exposed branches were nonetheless primed for an increased volatile response to gypsy moth feeding. Based on the amount of gypsy moth damage (approx. 2 cm^2) inflicted in HIPV-exposed branches (Fig. 4), we would expect a volatile emission rate of ~500 ng/g/h (based on Fig. 2). Instead, the observed amount was 1,314 ng/g/h, or an emission rate more than 2.5 times higher than that expected. Whether the full HIPV blend is required or whether specific compounds in the blend are responsible for the observed priming remains unclear. Corn seedlings previously exposed to HIPVs from neighboring plants induced greater volatile emissions than unexposed plants, a response also primed by (*Z*)-3-hexenyl acetate (Engelberth et al. 2004). In our study, (*Z*)-3-hexenyl acetate was emitted following herbivory and mechanical damage but not MeJA applications, and thus might represent a non-specific response in *V. corymbosum* due to physical damage to leaves. Despite the lack of specificity in its release, (*Z*)-3-hexenyl acetate activates plant defenses and has priming activity (Engelberth et al. 2004; Ruther and Kleier 2005; Kost and Heil 2006).

Responding to HIPVs by an increase in volatile emissions in the absence of herbivores might incur ecological as well as physiological costs, because HIPVs might provide unreliable information to the herbivores'

natural enemies. Instead, a more adaptive strategy might be for plants to prime themselves for an increased volatile response after exposure to HIPVs. This increased rate of volatile emission from HIPV-exposed leaves might serve as an indirect defense by preferentially attracting the natural enemies of herbivores (e.g., Ton et al. 2007). In addition, increased volatile emissions from HIPV-exposed leaves might serve a direct defense role since several volatiles induced by gypsy moth in *V. corymbosum*, including linalool and farnesenes, can have repellent effects on caterpillars (Markovic et al. 1996).

In summary, our results show that leaves from damaged *V. corymbosum* branches transmit information through volatiles that minimize insect feeding damage and prime volatile emissions in the receiving leaves of adjacent branches. These results are consistent with those from Karban et al. (2006) who found that sagebrush branches typically do not exchange signals via vascular connections but instead use external signals to activate resistance. The hypothesis that plants use airborne signals to transfer information to other parts of plants at risk of herbivore attack was first proposed by Farmer (2001), and Orians (2005) later expanded this hypothesis by suggesting that high architectural complexity within many plants might necessitate such external wound signaling. Our results combined with those by Karban et al. (2006), Heil and Silva Bueno (2007), and Frost et al. (2007) provide strong evidence that HIPVs play a role in intra-plant signaling. Indeed, external signaling via volatiles appears particularly important among branches of shrubs such as blueberries, where mobile caterpillars may be able to move relatively short distances among branches and evade induced defenses. Previous studies showed that HIPVs increase and/or prime defenses in neighboring plants (see review by Frost et al. 2008b). However, the ecological role of HIPVs in plant-to-plant communication remains unclear because the receiver plant often has to be in close proximity to the emitter plant (within 15 cm; Karban et al. 2003). In our study, the HIPV-emitting and -receiving branches were 10–15 cm apart; this distance is similar to that found between branches in natural settings (C.R.-S., personal observation). Thus, the effects of HIPVs in intra-plant signaling in blueberries likely occur also in nature, as shown in other systems (Karban et al. 2006; Heil and Silva Bueno 2007). This study provides compelling evidence for the role of HIPVs in intra-plant signaling and further demonstrates the multi-functional effects of HIPVs.

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Constitutive and Induced Activities of Defense-Related Enzymes in Aphid-Resistant and Aphid-Susceptible Cultivars of Wheat

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Abstract Phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), and peroxidase (POD) are considered important biochemical markers in host plant resistance against pest insects. Constitutive activity of these enzymes was analyzed in resistant and susceptible wheat cultivars against cereal aphid *Sitobion avenae* (F.) at various developmental stages, i.e., tillering, stem elongation, flag leaf, and ear. Following aphid infestation, the activity of these enzymes was determined at the flag leaf and ear stages. Resistant cultivars exhibited greater constitutive PAL activity than susceptible ones at the tillering, stem elongation, and flag leaf stages. Aphid infestation enhanced levels of PAL activity in the flag leaf and ear stages in both resistant and susceptible cultivars. Constitutive PPO activity was higher in the resistant cultivars at all developmental stages. Aphid infestation induced increases in PPO activity in the flag leaf and ear stages of one susceptible cultivar, whereas induction in resistant cultivars was weaker. Resistant cultivars showed greater constitutive POD activity in the tillering, stem elongation, and flag leaf stages, while aphid infestation induced POD activity in all cultivars, especially in susceptible ones. The potential role of PAL, PPO, and POD in wheat defense against aphid infestation is discussed.

Keywords Defense-related enzymes · Peroxidase · POD · Phenylalanine ammonia-lyase · PAL polyphenol oxidase · PPO · *Sitobion avenae* · Wheat

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Introduction

Host plant resistance to herbivores can suppress herbivore population densities and offers a promising approach for managing insect pests in a sustainable, economical, and environmentally safe manner (Heng-Moss et al. 2004). Plant secondary metabolites play an important role in plant defense against insects and pathogens (Cai et al. 2004), the levels of which are often mediated by defense-related enzymes, such as phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), and peroxidase (POD; Carver et al. 1992; Rosenthal and Berenbaum 1992; Bi and Felton 1995; Bi et al. 1997a, b, c).

PAL is a key enzyme in the phenylpropanoid pathway that converts phenylalanine into *trans*-cinnamic acid. *Trans*-cinnimaic acid can be further hydroxylated and methylated to produce compounds (i.e., coumaric, caffeic, and ferulic acids) that are toxic to herbivores and pathogens (Cole 1984; Leszczynski et al. 1989; Verpoorte and Alfermann 2000; Morelló et al. 2005; Wang et al. 2006). PPO and POD are oxidases that catalyze the formation of quinones from various phenolic precursors (Hildebrand et al. 1986; Mayer 1987; Felton et al. 1989; Duffey and Stout 1996).

PAL, PPO, and POD are associated with plant defense against insects (Felton et al. 1992; Stout et al. 1999; Chaman et al. 2001, 2003; Ni et al. 2001). However, little is

Table 1 Total constitutive protein contents (milligram per gram fw) of four wheat cultivars without aphid feeding at tillering and stem elongation stages

Stage	KOK1679	L1	Beijing 837	Beijing 411
Tillering	1.000±0.009 d	1.865±0.035 c	3.164±0.017 b	3.400±0.023 a
Stem elongation	0.812±0.003 c	1.950±0.030 b	4.060±0.052 a	4.099±0.054 a

Means in a row followed by different letter are significantly different at $P<0.05$

known about the dynamic changes of these enzymes during plant development and the influence of aphid infestation on their activity in resistant vs. susceptible cultivars, especially in wheat. The present research was initiated to investigate the constitutive and induced activity of these enzymes in *Sitobion avenae*-resistant and *S. avenae*-susceptible wheat cultivars at various developmental stages.

Methods and Materials

Plants and Insects Wheat cultivars KOK1679, L1, Beijing 411, and Beijing 837 were grown in 2003 and 2004 at the Science Park of China Agricultural University, Beijing, China. Wheat seeds were sown 20 cm apart in a 2-m single-row plot on October 14, 2003. Each plot was used as one replicate and covered with white nylon mesh to protect plants from insects and mechanical damage during the growth.

KOK1679 and L1 cultivars are resistant and repellant to the cereal aphid species *S. avenae*, while Beijing 411 and Beijing 837 are susceptible and attractive to this species (Liu et al. 2001). In addition, both KOK1679 and L1 have antibiotic properties against *S. avenae* compared to Beijing 411 and Beijing 837 (Cai et al. 2004 and unpublished data).

S. avenae nymphs and adults were collected from field-grown wheat and maintained on the aphid-susceptible wheat cultivar, Beijing 411, under field conditions. Insects over one generation were used for the experiment. When the unfolded flag leaf and ear emerged in 49–56 and 58–60 of Zadoks code (Zadoks et al. 1974), 20 flag leaves or ears from each experimental plot were selected and evenly divided for experimental and control treatments. Ten nymphs (second to third instar) were released onto each flag leaf and ear in each treatment. The flag leaves and ears were covered with nylon mesh cages (15×4 cm) to prevent the aphids from escaping (Rafi et al. 1996; Argandona et al. 2001). The experiments were arranged in a randomized complete block design, and each treatment was replicated three times.

Sample Collection A total of 60 tillers from each plot were sampled randomly at various developmental stages to determine the constitutive enzyme activity in wheat tissue. Whole plants, including stems and leaves at Zodaks stage 21–23 and the upper 3–5 young leaves and stem at Zadoks stage 31–34, were sampled (Zadoks et al. 1974). Flag leaves at stage 49–56 and ears at stage 58–60 also were sampled. Flag leaves and ears of all the treatments and controls were individually excised and collected 9 days after aphid release (Ni et al. 2001). Upon collection, samples were wrapped in aluminum foil and immediately dropped into liquid nitrogen for transport to a −80°C freezer before enzymatic analyses.

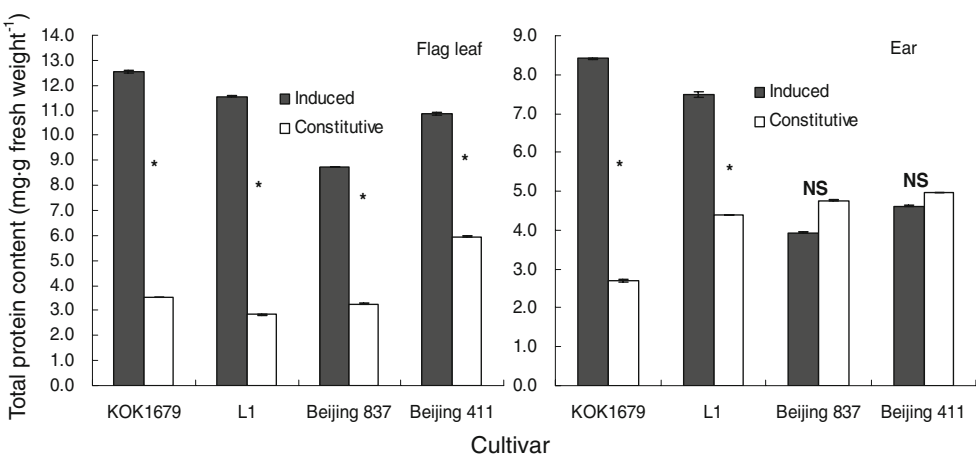

Fig. 1 Effect of *S. avenae* infestation on total protein content in flag leaf and ear stages of resistant and susceptible wheat cultivars. Significant difference at *$P<0.05$; *NS* no significant difference ($P>0.05$)

Table 2 Constitutive phenylalanine ammonia-lyase activity (dA_{290} per minute per milligram protein) of four wheat cultivars at tillering and stem elongation stages

Stage	KOK1679	L1	Beijing 837	Beijing 411
Tillering	1.590±0.166 a	1.512±0.287 a	0.736±0.135 b	0.836±0.069 b
Stem elongation	1.495±0.179 a	1.443±0.294 a	0.814±0.166 b	0.985±0.135 b

Means in a row followed by different letter are significantly different at $P<0.05$

Plant Protein and Enzyme Assays Plant tissue (100 mg) from each sample was ground in liquid nitrogen and homogenized in 20 mM HEPES buffer (500 µl; pH 7.2). The resulting mixture was centrifuged (Himac CR22E, Hitachi Koki Co. Ltd. Japan) at 10,000×*g* for 20 min at 4°C. The supernatant was used for analyses of total protein content and enzymatic activity.

The Bradford method (Bradford 1976) was used to determine total protein content in samples. Three aliquots (20 µl) of each sample were mixed with Bio-Rad reagent (180 µl, Bio-Rad, Richmond, CA, USA). Absorbance of the reaction mixture was determined at 595 nm, and protein content was determined from a standard curve established by using known quantities of bovine serum albumin (from Sigma Chemical Co.) and the above reagent.

PAL activity was determined following the method of Wang and Xue (1980) with enzyme extract (0.25 ml) in sodium borate buffer (1.5 ml, 30 mM; pH 8.8). The decrease of L-phenylalanine was monitored at 290 nm. PAL activity was estimated from dA_{290}. The assay was replicated three times.

POD activity was measured following a method previously described (Hildebrand et al. 1986; Hori et al. 1997). Enzyme extract (10 µl) was mixed with 1 ml of substrate containing hydrogen peroxide (10 µl; 30%, *w*/*w*), guaiacol (1 µl; 20 mM), and HEPES (100 µl; 200 mM, pH 7.0) in deionized water. POD activity was estimated from the increase in A_{470}. The measurement was repeated three times.

PPO activity was determined following the method of Hori et al. (1997). Enzyme extract (100 µl) was mixed with a solution containing catechol (500 µl; 1.6% *w*/*w*), HEPES buffer (100 µl; 200 mM, pH 6.0), and deionized water (800 µl). PPO activity was estimated from the increase in A_{470}. The analysis was repeated three times.

Statistical Analysis Analysis of variance (ANOVA) in SPSS (SPSS 11.0) was used to analyze PAL, POD, and PPO activity in wheat cultivars at various developmental stages. Pairs of treatment means were compared and separated ($\alpha=0.05$) by the least significant difference (LSD) procedure. Before the ANOVA and LSD procedure, PAL, POD, and PPO activity was transformed using the logarithm transformation (ln) to normalize the data.

Results

Protein Content Total protein content among wheat cultivars was significantly different at the different developmental stages (i.e., tillering, stem elongation, flag leaf, and ear stages), as well as at the flag leaf and ear stages in plants infested by aphids ($df=3, 11$, $F_{TS}=7294.6$, $F_{SES}=4887.1$, $F_{FL}=11040.8$, $F_{H}=9939.9$, $F_{AFL}=4883.9$, $F_{AH}=8267.3$, $P<0.001$). The constitutive protein content of

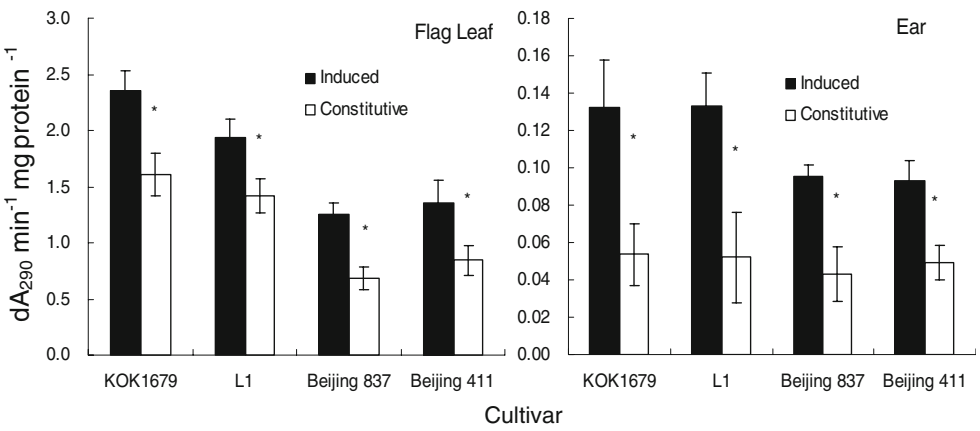

Fig. 2 Effect of *S. avenae* infestation on PAL activity in flag leaf and ear stages of resistant and susceptible wheat cultivars. Significant difference at *$P<0.05$; *NS* no significant difference ($P>0.05$)

Table 3 Constitutive polyphenol oxidase activity (dA_{470} per minute per milligram protein) of four wheat cultivars at tillering and stem elongation stages

stage	KOK1679	L1	Beijing 837	Beijing 411
Tillering	4.176±0.587 a	3.689±0.922 a	2.151±0.409 b	1.955±0.367 b
Stem elongation	4.594±0.348 a	4.108±0.487 a	3.106±0.632 b	3.078±0.414 b

Means in a row followed by different letter are significantly different at $P<0.05$

KOK1679 and L1 was lower than that of Beijing 411 and Beijing 837 at all stages, with the exception that the protein content of KOK1679 was higher than that of Beijing 837 at the flag leaf stage (Table 1, Fig. 1). However, after flag leaf and ear stages were infested by aphids, total protein content increased in flag leaves of all cultivars and in ears of KOK1679 and L1 ($P<0.05$; Fig. 1).

Defense-Related Enzymes KOK1679 and L1 exhibited greater constitutive PAL activity than Beijing 411 and Beijing 837 in the tillering and stem elongation stages (df=3, 11; F_{TS}=28.13, $P<0.01$; F_{SES}=6.53, P=0.02; Table 2). The constitutive PAL activity was also higher in KOK1679 and L1 than in Beijing 411 and Beijing 837 in the flag leaf (df=3, 11; F_{FL}=26.58, $P<0.001$), but not in the ear stage (Fig. 2). Aphid infestation strongly enhanced the levels of PAL activity in the flag leaf and ear stages of all four cultivars ($P<0.05$; Fig. 2).

Constitutive PPO activity was higher in KOK1679 and L1 than in Beijing 837 and Beijing 411 at all developmental stages (df=3, 11; F_{TS}=9.81, P=0.005; F_{SES}=7.34, P=0.011; F_{FL}=18.72, P=0.001; F_{E}=21.33, $P<0.001$; Table 3, Fig. 3). Aphid infestation caused significant increases in PPO activity in the flag leaf and the ear stages of Beijing 837 and Beijing 411 and in the ear stage of KOK 1679 (Fig. 3).

KOK1679 and L1 exhibited greater constitutive POD activity than Beijing 837 and Beijing 411 in the tillering and stem elongation stages (df=3, 11; F_{TS}=21.78, $P<0.001$; F_{SES}=15.28, P=0.001; Table 4). The constitutive POD activity was also higher in the flag leaf stage of KOK1679 and L1 than in Beijing 837 and Beijing 411 (df=3, 11; F_{FL}=15.79, P=0.001; Fig. 4). Aphid infestation boosted POD activity in all cultivars, especially in Beijing 837 and Beijing 411 ($P<0.05$; Fig. 4).

Discussion

Active defense in plants can be induced by both biotic and abiotic factors (Karban and Baldwin 1997; Kant et al. 2004). PAL, PPO, and POD are among the most important enzymes involved in the defensive responses of plants to insects and pathogens. PAL plays a key role in corn resistance to the maize sheath blight *Rhizoctonia solani* (Jin et al. 2003) as well as to *Helminthosporium maydis* and *Helminthosporium turcicum* (Wang and Xue 1980). Cole (1984) demonstrated that PAL activity is related to lettuce resistance to the lettuce root aphid *Pemphigus bursarius* (L). *S. avenae* infestation resulted in increased PAL activity in the flag leaf and ear stages of resistant and susceptible winter wheat cultivars (Ciepiela 1989; Havlickova et al. 1996). Increased PAL activity also was found in the leaves of *Brassica oleracea* seedlings infested by green peach aphid *Myzus persicae* (Sulzer; Zhang et al. 2005).

S. avenae-resistant wheat cultivars (i.e., KOK1679 and L1) had significantly higher levels of PAL activity than susceptible cultivars (Beijing 411 and Beijing 837) at the

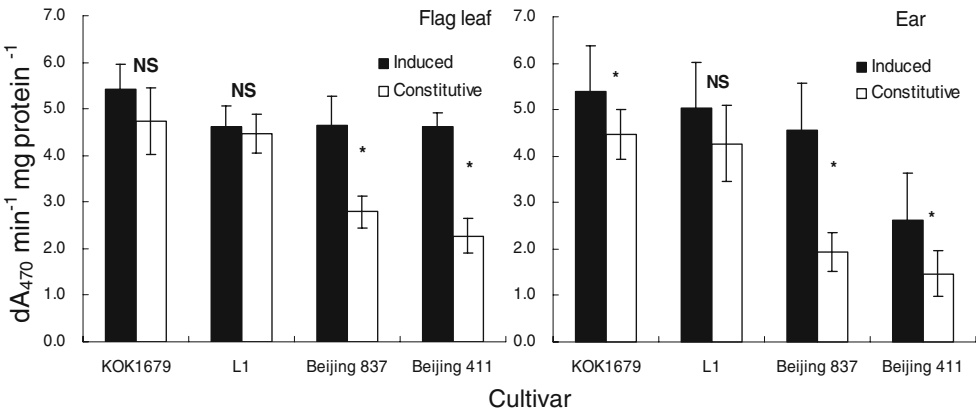

Fig. 3 Effect of *S. avenae* infestation on PPO activity in flag leaf and ear stages of resistant and susceptible wheat cultivars. Significant difference at *$P<0.05$; *NS* no significant difference ($P>0.05$)

Table 4 Constitutive peroxidase activity (dA_{470} per minute per milligram protein) of four wheat cultivars at tillering and stem elongation stages

Stage	KOK1679	L1	Beijing 837	Beijing 411
Tillering	68.643±1.059 a	65.058±1.037 a	24.649±1.067 b	24.616±1.025 b
Stem elongation	79.897±1.215 a	53.944±1.344 b	29.894±1.470 c	18.639±1.249 c

Means in a row followed by different letter are significantly different at $P<0.05$

tillering and stem elongation developmental stages. PAL activity in the flag leaf and ear stages of aphid-resistant cultivars increased more dramatically than that in the aphid-susceptible ones after *S. avenae* infestation, suggesting that the resistant cultivars may have a stronger capability to synthesize secondary metabolites related to insect susceptible ones.

Increased PPO activity in susceptible wheat can be induced by both aphids and methyl jasmonate (Leszczynski 1985; Boughton et al. 2006). Our study showed that constitutive PPO activity was higher in nutritional organs of resistant wheat cultivars compared to susceptible ones (Table 3). PPO activity in the ear and flag leaf stages of both resistant and susceptible cultivars increased when plants were infested with aphids; the increase was greater in susceptible cultivars (Fig. 3). However, Chrzanowski et al. (2003) reported that PPO activity in the ear stages of both resistant and susceptible spring wheat cultivars was decreased by *S. avenae* infestation. This discrepancy may be due, at least in part, to genetic differences in the wheat cultivars. Ni et al. (2001) reported that *Diuraphis noxia* feeding did not elicit changes in PPO activity in either resistant or susceptible cultivars of wheat or barley. This difference may be associated with the specific feeding behaviors exhibited by different aphid species, e.g., *D. noxia* feeding elicits leaf-chlorosis while *S. avenae* feeding does not.

Previous studies have demonstrated that POD activity can be enhanced by insects and elicitors such as ethephon and methyl jasmonate (Bi and Felton 1995; Bi et al. 1997a, b; Takahama and Oniki 1997; Kielkiewicz 1998). Buffalograss that is resistant to *Blissus occiduus* had higher POD activity compared to susceptible plants (Heng-Moss et al. 2004). Andres et al. (2001) reported that wheat root resistance to the cereal cyst nematode was associated with increased POD activity. POD activity in *B. oleracea* leaves infested with *M. persicae* increased significantly compared with control leaves (Zhang et al. 2005). *Macrosiphum euphorbiae* (Thomas) feeding induced greater POD activity in tomato leaves (Stout et al. 1998). Our results revealed that at the tilling and stem elongation stages, resistant wheat cultivars had a greater constitutive POD activity compared with susceptible cultivars, whereas at the flag leaf stage, the constitutive POD activities were similar between the resistant and susceptible cultivars (Table 4, Fig. 4). Also, aphid infestation of susceptible wheat cultivars at the flag leaf and ear stages induced a higher POD activity (Fig. 4). The differences in POD activities among the growing stages may be attributed to normal wheat plant growth (Ni et al. 2001).

In summary, *S. avenae*-resistant wheat cultivars had greater constitutive PAL, PPO, and POD activity at the tillering, stem elongation, and flag leaf stages of development. When the flag leaf and ear stages were attacked by aphids, PAL activity increased more dramatically in the resistant cultivars than in the susceptible ones. Aphid infestation increased activities of PPO and POD more dramatically in the susceptible cultivars than in the resistant ones.

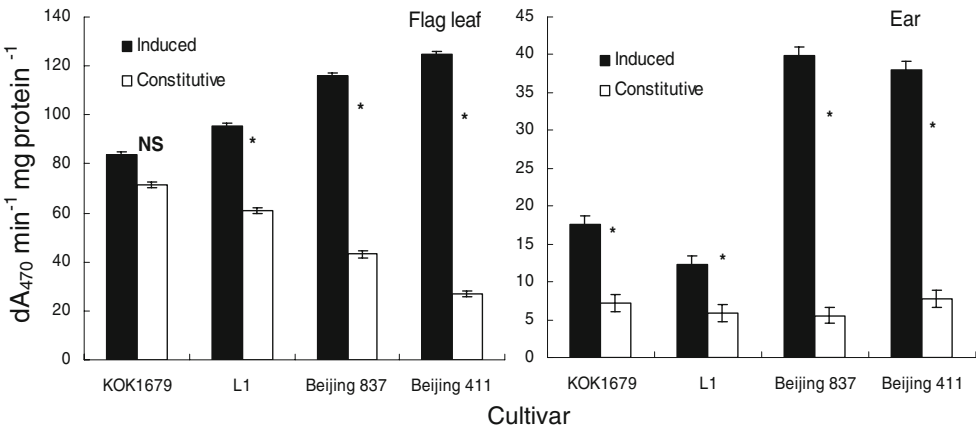

Fig. 4 Effect of *S. avenae* infestation on POD in flag leaf and ear of resistant and susceptible wheat cultivars. Significant difference at $*P<0.05$; *NS* no significant difference ($P>0.05$)

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Ecological Significance of Induction of Broad-Substrate Cytochrome P450s by Natural and Synthetic Inducers in *Helicoverpa zea*

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Abstract The polyphagous corn earworm *Helicoverpa zea* relies on cytochrome P450 monooxygenases with broad substrate specificities to cope with the wide diversity of phytochemicals it encounters among its numerous host plants. These enzymes also contribute to the ability of this insect to tolerate toxins from sources other than its hosts, including microbial and synthetic toxins. Although upregulation of xenobiotic-metabolizing P450s in some herbivorous insects is closely linked to host plant toxins, transcriptional and/or post-transcriptional regulation of detoxification in this polyphagous species also appears to be relatively unspecialized. Reverse transcription polymerase chain reaction and metabolic analyses indicate that rare and infrequently encountered phytochemicals, as well as synthetic substances, can enhance metabolic activity in an adaptive fashion against both natural and synthetic toxins.

Keywords Cytochrome P450 monooxygenases (P450s) · Plant–insect interactions · Transcriptional regulation · *Helicoverpa zea*

Introduction

Cytochrome P450 monooxygenases (P450s) catalyze the NADPH-associated reductive cleavage of oxygen to produce a functionalized product and water. The genes encoding these enzymes constitute one of the largest gene superfamilies known, and some insect genomes can contain upwards of 100 P450 genes (Tijet et al. 2001; Ranson et al. 2002; Feyereisen 2005; Strode et al. 2008; http://p450.sophia.inra.fr; http://drnelson.utmem.edu/CytochromeP450.html). The enormous proliferation of these proteins is, in part, a reflection of their functional diversity because, across their range, individual enzymes are capable of catalyzing a wide variety of transformations, including biosynthetic, bioactivation, and detoxification reactions (Fogleman et al. 1998; Feyereisen 1999, 2005; Scott 1999; Li et al. 2007). Especially relevant to the evolution of these loci and their encoded proteins in insects is that P450s mediate the metabolic detoxification of exogenous xenobiotics such as insecticides, mycotoxins, and plant toxins.

The diversity of reactions catalyzed by P450s presents an ecological challenge in terms of their transcriptional regulation. Upregulation of the appropriate isozymes is

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important in order to avoid the adverse consequences of bioactivation, rather than detoxification, of toxins. Little is known, however, about the mechanisms by which herbivorous insects regulate the multiplicity of P450s that detoxify host plant phytochemicals. In the black swallowtail *Papilio polyxenes*, the CYP6B1 protein is highly specialized for metabolizing linear furanocoumarins (Hung et al. 1997; Chen et al. 2002; Wen et al. 2003, 2006), the principal phytochemicals found in the apiaceous host plants of this oligophagous species. The promoter of the *CYP6B1* locus contains a strong positive regulatory element from −136 to −119 that is required for both basal and inducible transcription (Prapaipong et al. 1994; Petersen et al. 2003; Brown et al. 2005). This regulatory element appears to be specialized for furanocoumarins, consistent with the narrow host plant range of the insect. Similarly, the *CYP6B4* locus that encodes a P450 in the congeneric tiger swallowtail *Papilio glaucus* is transcriptionally induced in the midgut by dietary furanocoumarins (Hung et al. 1997; Li et al. 2001). Its promoter contains an overlapping EcRE/ARE/XRE-xan element similar to that used for basal and xanthotoxin-inducible expression of the *CYP6B1* promoter (McDonnell et al. 2004); this EcRE/ARE/XRE-xan element is necessary for CYP6B4 induction by xanthotoxin but not for its minimal basal expression. In addition to these elements, the *CYP6B4* and *CYP6B1* promoters also contain putative XRE-AhR elements identical to the aryl hydrocarbon response elements of many mammalian phase I detoxification genes (Hung et al. 1996). Transfections of the *CYP6B4* and *CYP6B1* promoters that contain EcRE/ARE/XRE-xan and XRE-AhR elements demonstrate that both are inducible by the furanocoumarin xanthotoxin as well as by benzo-α-pyrene (Prapaipong et al. 1994; Petersen et al. 2003; McDonnell et al. 2004; Brown et al. 2005), an aryl hydrocarbon combustion product that is widespread in terrestrial environments.

Transcriptional regulation of P450s by dietary constituents should be particularly challenging for broadly polyphagous species, which can encounter dozens of biosynthetically distinct toxins across their range. Given the diversity of P450s within an insect genome and the multiplicity of functions, including the differential potential to bioactivate or detoxify, induction of particular isoforms may actually enhance, rather than reduce, toxicity. As a broadly polyphagous herbivore, *Helicoverpa zea* encounters a wide range of phytochemicals in its >100 species of plant hosts. Inasmuch as many of its common hosts, including *Zea mays*, are susceptible to infection by *Aspergillus* fungi that produce aflatoxins, *H. zea* also routinely encounters these mycotoxins. Moreover, due to their ability to inflict economic damage on a diversity of crop plants, these insects are also frequent targets of synthetic insecticides. Previous studies have shown that certain widespread phytochemicals, including plant hormones and defense compounds (Li et al. 2002a, b; Zeng et al. 2007), can upregulate certain P450s in this species. Phytochemicals that are frequently (coumarin), occasionally (indole-3-carbinol), or rarely (xanthotoxin) encountered by *H. zea* larvae substantially reduced the toxicity of aflatoxin B1 (AFB1) to *H. zea*, despite the fact that certain *H. zea* P450s can bioactivate aflatoxins (Zeng et al. 2007). As well, diets that contain coumarin or xanthotoxin reduce the mortality rates of caterpillars exposed to the insecticides diazinon and carbaryl, indicating that consumption of plant chemicals can ameliorate toxicity of natural and synthetic toxins in this species by inducing P450s that detoxify these chemicals (Zeng et al. 2007).

For this study, we examined transcriptional regulation in this polyphagous species to test whether the frequency of evolutionary encounter, as indicated by origin (synthetic or natural) and distribution across plant families (unique to one or widespread), is predictive of the ability of compounds to induce transcriptional regulation of broadly substrate-specific P450s. Specifically, we compared the induction ability of phytochemicals that are extremely restricted in distribution (flavone, reported in *Primula malacoides* (Weller et al. 1953)); phytochemicals occurring in a number of genera in one or two families (khellin and visnagin, furanochromones restricted to the Apiaceae; imperatorin, a furanocoumarin restricted to the Apiaceae and Rutaceae); phytochemicals present in a wide range of plant families (rutin, a flavonoid glycoside present in >12 plant families); and a synthetic chemical (β-naphthoflavone). We also tested whether P450s that metabolize wide range of substrates including phytochemicals, mycotoxins (aflatoxins), and synthetic insecticides (diazinon, carbaryl, cypermethrin), are as likely to be upregulated by synthetic compounds as by naturally occurring compounds and whether extremely rare phytochemicals are as likely to induce generalized P450s as are widely distributed phytochemicals.

Methods and Materials

Chemicals and Insects AFB1, flavone, β-naphthoflavone (β-NF), rutin, khellin, and visnagin were obtained from Sigma (St. Louis, MO, USA) and imperatorin from Feinbiochemica (Heidelberg, Germany). Diazinon, carbaryl, aldrin, and α-cypermethrin were purchased from Chem Service (West Chester, PA, USA). Analytical grade dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific.

Larvae of *H. zea* were obtained from a colony maintained by the UIUC Department of Entomology, founded originally from individuals obtained from Abbott Laboratories (Chicago, IL, USA) and supplemented intermittently with wild-caught individuals from Champaign

County, IL. Larvae were individually reared on semisynthetic diet containing wheat germ (Waldbauer et al. 1984) and maintained in an insectary kept at 23–26°C with a photoperiod of 16-h L: 8-h D.

Heterologous Expression and Metabolism of Insecticides by CYP6B8, CYP6B27, and CYP321A1 Heterologous expression of P450s with house fly P450 reductase and *in vitro* insecticide metabolism assays for each enzyme were conducted as described in Wen et al. (2003) and Sasabe et al. (2004) with some modifications. Briefly, recombinant CYP6B8, CYP6B27, or CYP321A1 viruses were co-expressed with house fly P450 reductase virus in Sf9 cells at a multiplicity of infection ratio of 1:1, 1:1, and 2:2, respectively. Microsomes prepared from Sf9 cells infected with combinations of P450 and P450 reductase viruses were analyzed in *in vitro* metabolism assays as described in Rupasinghe et al. (2007). Duplicate reactions were set up, initiated with the addition of NADPH, and incubated at 30°C for 30 min. Reactions were stopped by adding 250 µl acetonitrile that contained the same amount of internal standard to normalize for procedural variations. Parathion was used as an internal standard for diazinon, diflubenzuron for carbaryl, and methoxychlor for both aldrin and α-cypermethrin. The final reactions were mixed by vortexing and microcentrifuged at full speed for 10 min, and the supernatant was passed through a 0.45-µM Alltech–Microspin filter (Alltech, Deerfield, IL, USA) by centrifugation at 5,000 rpm in an Eppendorf centrifuge for 5 min. The filtered supernatants were run on reverse phase high-performance liquid chromatography (XTerra 5-µm column, 150×4.6 mm) to analyze the proportion of substrate remaining. For analyses of diazinon and carbaryl, acetonitrile/water (55:45) was used as solvent and run at a flow rate of 1 ml/min. For analyses of aldrin and α-cypermethrin, acetonitrile/water (70:30) was used as solvent with a flow rate of 1.2 ml/min. To detect each chemical and its internal standard, UV monitoring was at 235 nm for diazinon, 280 nm for carbaryl, and 220 nm for both aldrin and α-cypermethrin. By using the solvent conditions listed above, the retention times for each chemical were 4.0 min for carbaryl, 5.2 min for methoxychlor, 8.6 min for diazinon, 8.9 min for diflubenzuron, 11.0 min for aldrin, 11.8 min for parathion, and 9.2 min for α-cypermethrin. P450 enzymatic activities are reported as substrate disappearance and expressed in nanomole substrate disappearance per minute per nanomole P450. Each metabolic assay was replicated in at least three biological replicates with each *in vitro* assay done in duplicate.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Gel Blot Analysis of P450 Transcripts from Corn Earworms Exposed to Inducers Fifth instar *H. zea* molted within a 12-h period and with a weight range from 170–270 mg/larva were randomly grouped. Larvae in each group of 20 were individually switched to diets containing inducers at a concentration of 0.1% (*w*/*w*) for flavone, β-NF, and rutin, 0.015% for khellin and visnagin, and 0.01% for imperatorin. These concentrations were selected because they represent the highest concentration that can be ingested without adverse effects on growth or development (data not shown). Each inducer was first dissolved in DMSO corresponding to about 0.2% diet weight (*v*/*w*) for even distribution. Larvae feeding on a diet that contained 0.2% DMSO served as the control. After larvae fed for 48 h, their midguts were dissected and cleaned in 0.1 M sodium phosphate buffer (pH 7.8). Total RNA from each group was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), treated with RQ DNase I (Promega, Madison, WI, USA), reisolated with TRIzol reagent, and finally suspended in sterile water and stored at –80°C.

RT-PCR gel blot Southern analysis was used to quantify the CYP6B8, CYP6B27, and CYP321A1 transcripts in midguts as described in Zeng et al. (2007), with actin primers included with one set of P450 primers in each reaction to minimize variation. RT-PCR Southern analyses were repeated two times with RNA samples prepared from midguts of larvae in two independent biological series.

Effects of Inducers on the Ability of H. zea to Tolerate Insecticides To test the effects of these inducers on *H. zea* challenged with insecticides, fifth instars that had molted within a 12-h period were provided for 48 h with diets containing the inducers flavone, β-NF, or rutin or with DMSO (as a control) as described. These caterpillars then were exposed to topically applied insecticides corresponding to 300 µg diazinon, 100 µg carbaryl, or 150 ng α-cypermethrin per larva. These diagnostic doses were determined in a series of preliminary bioassays. Following topical application of insecticides in 3 µl acetone, the caterpillars that were still feeding were kept in an incubator at a temperature of 28°C and a photoperiod of 16-h L: 8-h D. Mortality was checked periodically 4 h after insecticide application, with caterpillars considered dead if they were unable to right themselves when on their backs. These bioassays were replicated at least three times with 20 larvae tested for each treatment.

Effects of Inducers on H. zea Ability to Tolerate AFB1 To test the effects of natural and synthetic inducers on *H. zea* challenged with AFB1, caterpillars concurrently exposed to AFB1 and an inducer were compared with those exposed to either AFB1 or the inducer alone. In separate experiments, either fourth or fifth instars that had molted within a 12-h period were randomly distributed among four groups, with 20 caterpillars in each group. Caterpillars were

switched individually to 30-ml plastic cups that contained diets supplemented with (1) DMSO, (2) an inducer, (3) AFB1, or (4) an inducer and AFB1. The final concentrations of inducers and AFB1 in the diet (*w*/*w*) were 0.1% for flavone and β-NF, 0.015% for visnagin, 0.01% for imperatorin, and 1 μg/g for AFB1. All were prepared in DMSO so that they could be dispersed evenly in the diet at a final concentration of 0.2% (*v*/*w*).

Caterpillars feeding on these different diets were individually reared in an insectary at 23–26°C with a photoperiod of 16-h L: 8-h D. Pupation rates and pupal weight were examined as described in Zeng et al. (2007), with each set of bioassays replicated three times.

Statistical Analyses All data were evaluated by one-way factorial analysis of variance (ANOVA) with treatment differences among means tested at $P = 0.05$ by Tukey post-hoc test. All data are means from three replicates with 20 caterpillars/treatment.

Results

Heterologous Expression and P450 Activities Previous studies have determined that *H. zea* CYP6B8 and CYP321A1 have a broad range of substrates. CYP6B8 metabolizes phytochemicals from at least four biosynthetic classes (including the flavonoids quercitin, rutin, and flavone, the furanocoumarin xanthotoxin, the glucosinolate precursor indole-3-carbinol, and the phenolic chlorogenic acid) and at least four synthetic chemicals (β-naphthoflavone, the insecticides diazinon, cypermethrin, and aldrin; Li et al. 2004; Rupasinghe et al. 2007), whereas CYP321A1 metabolizes furanocoumarins (xanthotoxin, angelicin), a mycotoxin (aflatoxin B1), and the same four synthetic compounds (Sasabe et al. 2004, Rupasinghe et al. 2007). Heterologous expression of CYP6B8 or CYP321A1 simultaneously with house fly P450 reductase has allowed us to add to the known substrate specificities of these two enzymes. Heterologous expression of the related CYP6B27 protein with house fly P450 reductase has permitted side-by-side activity comparisons of these three P450s (Table 1). All three enzymes can metabolize four different classes of insecticides: cyclodienes (aldrin), pyrethroids (α-cypermethrin), organophosphates (diazinon), and carbamates (carbaryl). The metabolic activities of CYP6B8 and CYP6B27, which are closely related (88% amino acid identity; Li et al. 2004), are higher than that of CYP321A1 for all insecticides except for carbaryl, against which all three enzymes have similar activities. The metabolic activities of the CYP6B8 and CYP6B27 enzymes are similar, with the rank order aldrin > α-cypermethrin ≈ diazinon > carbaryl.

Table 1 Metabolic activities of CYP6B8, CYP6B27 and CYP321A1 against insecticides

Substrate	metabolic activity ± SE (nmol/min/nmol P450)		
	CYP6B8	CYP6B27	CYP32141[a]
Aldrin	14.33 (1.85)	18.14 (0.54)	5.28 (0.85)
Diazinon	2.33 (0.12)	2.31 (0.08)	1.35 (0.21)
Carbaryl	0.28 (0.06)	0.27 (0.03)	0.26 (0.07)
α-cypermethrin	2.44 (0.65)	2.01 (0.38)	1.60 (0.33)
Aflatoxin B1	ND[a]		2.60 (0.20)

ND not detectable
[a] From Niu et al. 2008

Effects of Inducers on P450 Transcript Accumulation Although the six inducers differentially upregulate the transcription of the *CYP6B8* and *CYP321A1* loci, there is no obvious pattern whereby the natural compounds are superior to synthetic compounds as inducers, nor is there any consistent pattern demonstrating that ubiquitous phytochemicals are more effective inducers than are rarely encountered phytochemicals. Compared to the transcript levels in midguts of control larvae feeding on a diet

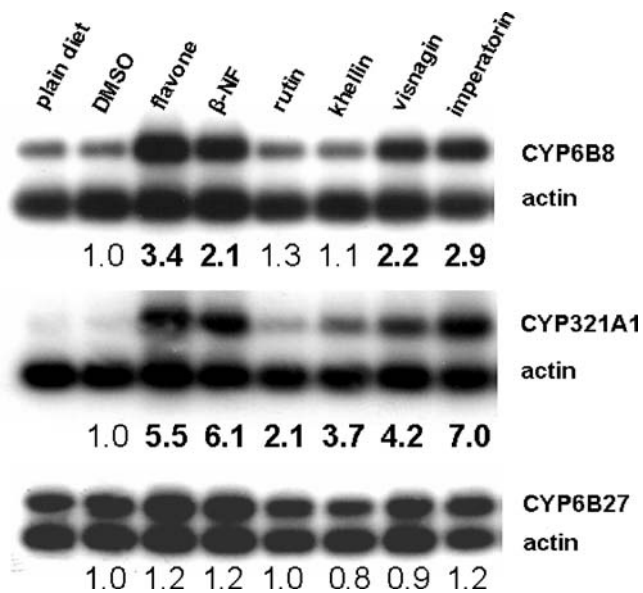

Fig. 1 P450 transcript induction in the midguts of fifth instar *H. zea* by synthetic and natural inducers. Newly molted fifth instars were fed an artificial control diet, a diet containing DMSO (0.2% *w*/*w*), or a diet containing an inducer dissolved in DMSO and incorporated into the diet. RT-PCR products detected with gene-specific primers and probes correspond to: (*1*) control unamended diet and diets containing (*2*) 0.2% DMSO, (*3*) 0.1% flavone, (*4*) 0.1% β-NF, (*5*) 0.1% rutin, (*6*) 0.015% khellin, (*7*) 0.015% visnagin, (*8*) 0.01% imperatorin

containing 0.2% DMSO, the *CYP321A1* transcript levels from midguts of larvae feeding on diets containing any one of the six inducers were higher, ranging from 2.1-fold for rutin to 7.0-fold for imperatorin (Fig. 1). Relative to larvae on diets lacking inducers, *CYP6B8* transcript levels were induced for larvae feeding on diets containing flavone (3.4-fold), β-NF (2.1-fold), visnagin (2.2-fold), and imperatorin (2.9-fold). In contrast, despite the close relationship of its amino acid sequence with CYP6B8, transcription of the *CYP6B27* locus was not apparently induced by any of the compounds tested.

Effects of Inducers on the Ability of H. zea to Tolerate Insecticides and Mycotoxins Irrespective of whether prior exposure was to synthetic (β-NF) or to natural inducers (flavone, rutin), *H. zea* larvae experienced significantly lower mortality upon subsequent exposure to α-cypermethrin or diazinon (Fig. 2). In these trials, flavone had the most dramatic effect in decreasing the mortality of *H. zea* exposed to organophosphates (as represented by diazinon), carbamates (carbaryl), or pyrethroids (α-cypermethrin). Compared to approximately 90% mortality in control corn earworms challenged with diazinon, no mortality was observed for diazinon-treated larvae that had ingested flavone diet before insecticide treatment, and all of these larvae pupated. Similarly, concurrent exposure of either fourth (Fig. 3) or fifth instar (Fig. 4) corn earworm to aflatoxin B1 along with synthetic (β-NF) or natural plant allelochemicals (flavone, visnagin, and imperatorin) significantly improved larval performance in comparison with larvae exposed only to aflatoxin B1, as demonstrated by pupation rates and pupal weights (Figs. 3 and 4).

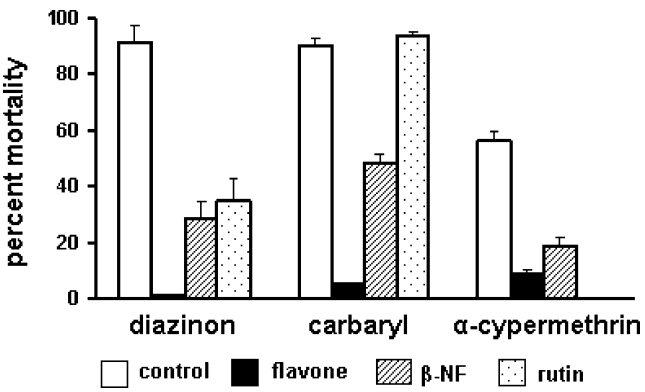

Fig. 2 Effects of inducers on *H. zea* tolerance of insecticides. Twenty newly molted fifth instar *H. zea* were fed either a control diet or a diet containing one of three inducers (0.1% flavone, β-NF, or rutin) for 48 h. These larvae were then topically treated with one of three insecticides (300 μg diazinon, 100 μg carbaryl, or 150 ng α-cypermethrin per larva) and mortality was tabulated 18 h post-treatment

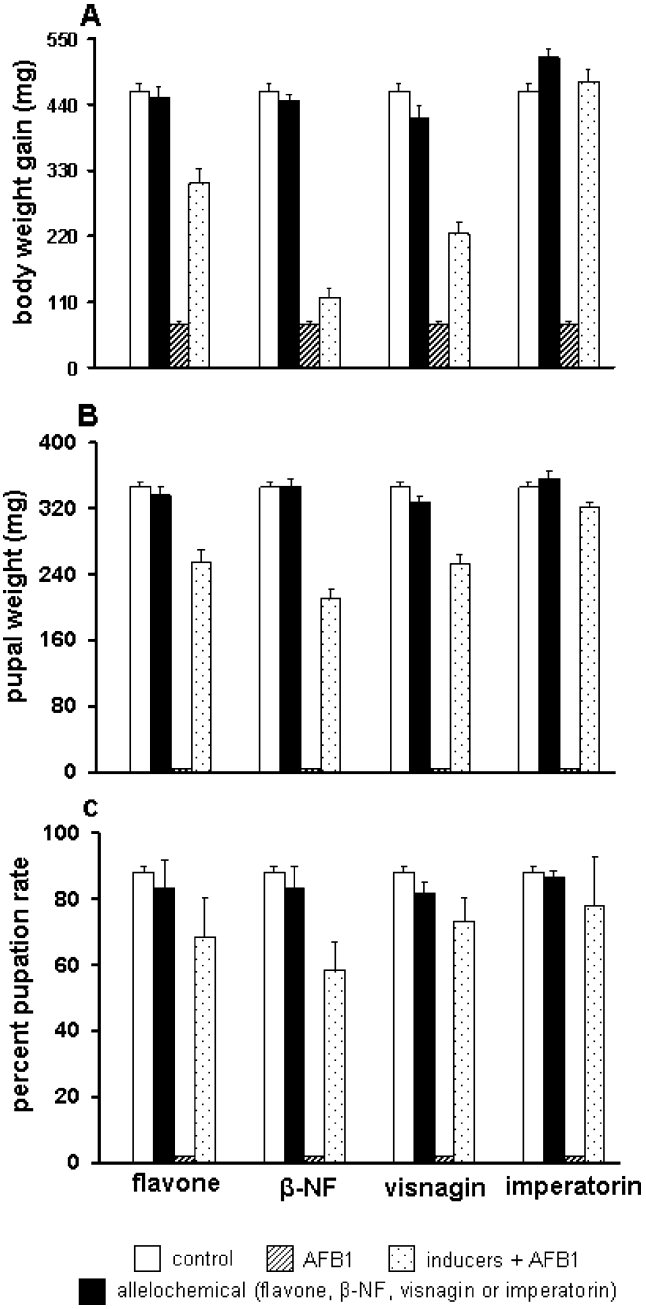

Fig. 3 Effects of inducers on fourth instar *H. zea* tolerance of aflatoxin B1. Newly molted fourth instar *H. zea* fed artificial diet containing DMSO (0.2% *w*/*w*), an inducer, AFB1, or a combination of an inducer and AFB1. The final concentrations of inducers and AFB1 in the diet (*w*/*w*) were 0.1% for flavone and β-NF, 0.015% for visnagin, 0.01% for imperatorin, and 1 μg/g for AFB1. **A** body weight gain in 6 days; **B** pupal weight; **C** pupation rate

Discussion

H. zea evidently relies on multiple P450s with broad substrate specificity to metabolize a wide variety of both natural and synthetic substrates that include plant allelo-

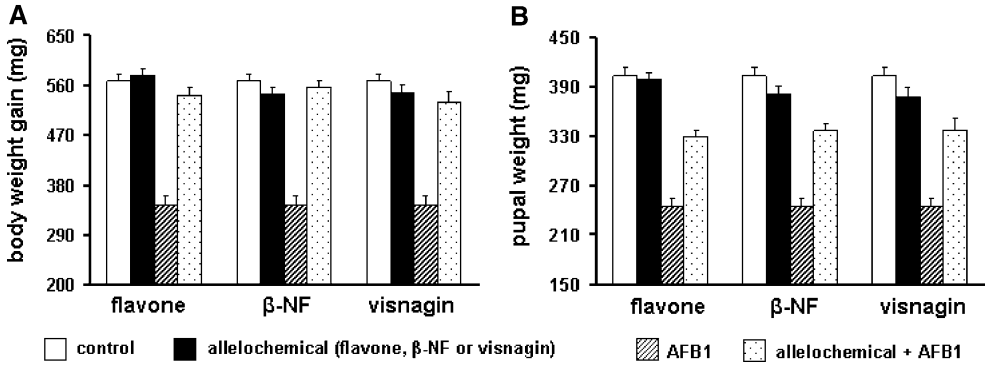

Fig. 4 Effects of inducers on fifth instar *H. zea* tolerance of aflatoxin B1. Newly molted fifth instar *H. zea* fed artificial diet containing DMSO (0.2% *w*/*w*), an inducer, AFB1, or a combination of an inducer and AFB1. The final concentrations of inducers and AFB1 in the diet (*w*/*w*) were 0.1% for flavone and β-NF, 0.015% for visnagin, 0.01% for imperatorin, and 1 μg/g for AFB1. **A** body weight gain in 3 days; **B** pupal weight

chemicals, insecticides, and mycotoxins. Even though the number of P450s examined in this species was small, these loci varied in their respective inducibilities. That the P450 with the overall lowest level of activity toward this array of substrates, CYP321A1, showed the highest level of inducibility resembles the pattern observed in the oligophagous *P. polyxenes*, in which CYP6B3, with overall lower levels of activity against furanocoumarin substrates, displays a greater induction response than does CYP6B1, with higher levels of activity against those same substrates (Hung et al. 1995). In contrast with *P. polyxenes*, which is characterized by specialized P450s that are tightly regulated by promoter elements responsive to specific host plant constituents (Prapaipong et al. 1994), the P450s in *H. zea* are regulated by a broad range of chemicals. Synthetic substances are equal to or more effective than widespread phytochemicals at upregulating transcripts for detoxification enzymes, and even rare phytochemicals, which may never be encountered by this insect, are effective at inducing transcription.

The specificity of transcriptional responses to inducers has been examined in relatively few insect systems, so establishing a general pattern is difficult. Le Goff et al. (2006) used DNA microarray hybridization and real-time PCR to examine P450 transcript accumulation patterns in response to two synthetic inducers (phenobarbital and atrazine) in *Drosophila melanogaster*. These authors found that only 11 of 86 P450 loci were induced by phenobarbital and only seven by atrazine. Three of these loci (CYP6A2, CYP6A8, CYP12D1) are associated with resistance to insecticides, suggesting that broad specificity (and the ability to metabolize evolutionarily novel substrates) is associated at least in some cases with a lack of specificity in transcriptional and/or post-transcriptional regulation, although the strength of that association is difficult to assess in view of the absence of information on the substrate profiles of other P450s in this genome. In contrast, Willoughby et al. (2006), also by using microarray analysis with *D. melanogaster*, failed to find a detectable induction response of detoxification enzymes, including P450s, to the insecticides spinosad, diazinon, nitenpyram, lufenuron, and dicyclanil; DDT exposure led to minimal induction of a single P450. The phytochemical caffeine, which likely is not naturally encountered by *D. melanogaster*, induced a variety of P450s under the same experimental conditions, as did the synthetic drug phenobarbital. In another dipteran, *Aedes aegypti*, Poupardin et al. (2008) used the *Aedes* Detox Chip microarray and quantitative real-time PCR to examine the inducibility of genes that encode detoxification enzymes by a variety of environmental xenobiotics, including insecticides. They found a selective response in fourth instar larvae, with only seven of 168 P450 genes in the genome responsive to any of the agents tested.

Based on a limited number of studies, then, it appears that transcriptional and/or post-transcriptional regulation of P450s in polyphagous insects is opportunistic and evolutionarily flexible. Indeed, Amichot et al. (1998) documents variation in induction responses among strains of *D. melanogaster*. Given the overall importance of P450s in such phenomena as the evolution of insecticide resistance and host shifts in polyphagous herbivores, understanding the mechanisms that provide that flexibility might well provide novel targets for sustainable pest management.

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Enzyme Induction as a Possible Mechanism for Latex-Mediated Insect Resistance in Romaine Lettuce

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Abstract Plant latex is a known storehouse of various secondary metabolites with demonstrated negative impact on insect fitness. A romaine lettuce cultivar, "Valmaine", possesses a high level of latex-mediated resistance against the banded cucumber beetle, *Diabrotica balteata* LeConte (Coleoptera: Chrysomelidae), compared to a closely related cultivar "Tall Guzmaine". Latex from damaged Valmaine plants was much more deterrent to adult *D. balteata* feeding than latex from undamaged plants when applied to the surface of artificial diet under choice conditions; no such difference was found in choice tests with latex from damaged and undamaged Tall Guzmaine plants. The intensities of whiteness and browning were significantly higher in Valmaine latex than in Tall Guzmaine latex. The activities of three enzymes (phenylalanine ammonia lyase, polyphenol oxidase, and peroxidase) significantly increased over time in latex from damaged Valmaine plants (i.e., 1, 3, and 6 days after feeding initiation), but they remained the same in Tall Guzmaine latex. The constitutive levels of phenylalanine ammonia lyase and polyphenol oxidase also were significantly higher in Valmaine latex than in Tall Guzmaine latex. These studies suggest that Valmaine latex chemistry may change after plant damage due to increased activity of inducible enzymes and that inducible resistance appears to act synergistically with constitutive resistance against *D. balteata*.

Keywords Banded cucumber beetle · Chrysomelidae · Coleoptera · *Diabrotica balteata* · Enzymes · Induced defense · Latex · Laticifers · *Lactuca sativa* · Oxidative browning · Peroxidase · Phenylalanine ammonia lyase · Plant defense · Polyphenol oxidase

Introduction

Lettuce, *Lactuca sativa* L., is an important vegetable crop grown throughout the world (Ryder 1998). Lettuce growers suffer huge economic losses due to various insect pest infestations because of the high cosmetic standards demanded by consumers (Palumbo et al. 2006). The romaine lettuce cultivar, "Valmaine", exhibits a high level of resistance against various insect pests, including the leafminer, *Liriomyza trifolii* (Burgess; Nuessly and Nagata 1994), banded cucumber beetle, *Diabrotica balteata* LeConte (Coleoptera: Chrysomelidae; Huang et al. 2002), and two moths, *Trichoplusia ni* (Hübner) and *Spodoptera exigua* (Hübner; Sethi et al. 2006). Valmaine's resistance would be useful in an integrated pest management program; however, this cultivar is not popular among growers because of its susceptibility to thermodormancy, premature bolting, lettuce mosaic virus, and corky root rot (Guzman 1986). Plant breeders have attempted to improve the horticultural characteristics of Valmaine through breeding, but unfortunately, the horticulturally improved and currently used cultivar, "Tall Guzmaine", lost resistance to insects during the improvement process (Sethi et al. 2006). Tall Guzmaine was selected from a cross between "Short

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Guzmaine" and "Parris White", and Short Guzmaine was a selection from a cross between Valmaine and Florida 1142 (Guzman 1986).

Our previous research revealed that latex extracted from undamaged Valmaine plants and applied to the surface of artificial diet deterred feeding of adult *D. balteata*, whereas latex from Tall Guzmaine did not (Sethi et al. 2008). We hypothesized that constitutive levels of compounds in Valmaine's latex may explain the feeding deterrence shown by Valmaine against multiple insect species. Furthermore, Valmaine plants previously damaged by feeding *D. balteata* showed an increased localized resistance to *D. balteata* feeding compared to undamaged plants, suggesting the involvement of an inducible mechanism of resistance (Huang et al. 2003). Tall Guzmaine showed no such inducible resistance.

Latex is an aqueous suspension or solution of complex mixtures of molecules found in specialized secretory cells of plants known as laticifers (Pickard 2008). Laticifers possess high metabolic activity; in addition to synthesizing numerous molecules (lipids, sugars, and proteins) required to achieve basic physiological functions, laticifers also are known to synthesize and store diverse secondary metabolites in appreciable amounts in latex (El Moussaoui et al. 2001). Many defensive compounds with demonstrated negative impact on insect fitness are stored in latex (Evans and Schmidt 1976; Matile 1976; Nishio et al. 1983; Rees and Harborne 1985; Konno et al. 2004, 2006; Ramos et al. 2007). Latex whiteness arises due to differences in the refractive indices of the dispersing particles (terpenoids, alkaloids, tannins, proteins, and sterols) and the dispersing medium (Van Die 1955; Esau 1965; Archer et al. 1969; Groeneveld 1976; Fox and French 1988). Various organic compounds, such as terpenoids and phenolics, have been reported in latex of *Lactuca* spp. (Cole 1984; Dupont et al. 2000; Sessa et al. 2000), but their biological activity against insects has not been reported in lettuce. Their defensive role as phytoalexins, however, has been reported against plant pathogens (Bennett et al. 1994).

Activity of defense-related enzymes, such as phenylalanine ammonia lyase (PAL) and polyphenol oxidase (PPO), is much higher in the laticifers than in the leaves of rubber tree, *Hevea brasiliensis* H.B.K. (Kush et al. 1990; Wititsuwannakul et al. 2002). Wounding of laticifers is also known to induce various defense-related enzymes in latex of papaya (Azarkana et al. 2004), fig tree, *Ficus carica* L. (Kim and Mullin 2003), rooster tree, *Calotropis procera* Ait. (Freitas et al. 2007), and Albanian spurge, *Euphorbia characias* L. (Mura et al. 2005, 2007). Thus, alteration in the constituents of latex upon insect damage can enhance its defensive properties.

Latex commonly turns brown after exuding from plant wounds. Browning is due to contact with oxygen of reactive *ortho*-quinones produced when polyphenol oxidase contacts its chemical substrates in plant tissues damaged during insect feeding (Constabel and Ryan 1998). The reactive *ortho*-quinones readily form alkylated amino acids, which ultimately result in protein modification, cross-linking, and precipitation. This protein modification significantly impacts insect pests by preventing efficient digestion and assimilation of nitrogen (Duffey and Stout 1996).

The purpose of this study was to determine whether the previously reported induced resistance in Valmaine lettuce (Huang et al. 2003) is also mediated through latex, as is Valmaine's constitutive resistance (Sethi et al. 2008). If beetle damage did indeed trigger an increase in feeding deterrence of latex, our next objective was to determine whether that increase in deterrence was correlated with an increase in activities of inducible enzymes. Hence, *D. balteata* adults were given choices between artificial diets treated topically with latex from damaged and undamaged plants within the same cultivar (Valmaine or Tall Guzmaine) to look for changes in latex deterrence after beetle damage. We then compared the induction over time of defense-related enzymes, in particular PAL, PPO, and peroxidase (POX), in latices of resistant Valmaine and susceptible Tall Guzmaine with and without *D. balteata* damage. We then studied the relationship between the activities of these enzymes and the weight gain of the beetles fed on the plants. We also investigated change in color characteristics of latex (whiteness and browning) from damaged and undamaged plants in response to beetle damage.

Methods and Materials

Plants Seeds of romaine lettuce cultivars Valmaine and Tall Guzmaine (provided by R. T. Nagata, Everglades Research and Education Center, University of Florida, Belle Glade, FL, USA) were germinated overnight on moistened filter paper. Germinated seeds were planted in transplant trays filled with Metro-Mix 200 (Sun Gro Horticulture, Bellevue, WA, USA) in a glasshouse with ambient light at a mean temperature of 27°C (21–30°C) and a mean relative air humidity (RH) of 71% (53–89% RH). Healthy seedlings were transplanted 2 weeks later into plastic pots (12.5× 9 cm, diameter × depth). Plants were watered daily and fertilized with 15 ml of Peters 20-20-20 solution (10 g/l; W. R. Grace, Fogelsville, PA, USA) every week. Six-week-old lettuce plants (9–10 true-leaf stage) were used for experiments. Bush lima bean seeds, *Phaseolus lunatus* L. (Fabaceae), of the cultivar Fordhook 242 (Illinois Foundation Seeds, Champaign, IL, USA) were planted in transplant trays filled with Metro-Mix 200 and fertilized with the same solution used for lettuce plants.

Insects The colony of *D. balteata* was started from adults collected in 2003 from weeds [spiny amaranth, *Amaranthus spinosus* L. (Amaranthaceae), and primrose willow, *Ludwigia peruviana* (L.) H. Hara (Onagraceae)] in Belle Glade, FL, USA. Wild adults were added to the established colony in 2005 and 2006 to increase genetic diversity. Larvae were reared on the roots of corn seedlings, and adults were fed on lima bean leaves and sweet potato tubers (Huang et al. 2002). Newly emerged adults were maintained at 27±1°C in an incubator at a photoperiod of 14:10 h ratio of light to dark and supplied with water through a dental wick placed in a container with a hole in the lid. Adults less than 48 h old were used for experiments.

Artificial Diet Freshly made southern corn rootworm, *Diabrotica undecimpunctata howardi* Barber, artificial diet (Bio-Serv, Frenchtown, NJ, USA) was prepared according to Sethi et al. (2008) and used in all experiments. One-centimeter-thick disks were punched out from cooled artificial diet with a 1.5-cm-diameter cork borer.

Color Characteristics of Latex from Damaged and Undamaged Plants Thirty plants of each cultivar were placed individually in cylindrical plastic cages (18.5 cm diameter× 61.0 cm height) with three screened ventilation holes. Two male–female beetle pairs were placed into half (15 plants) of the cages of each cultivar; the other 15 plants of each cultivar were used as undamaged checks. Latex was collected from plants 1, 3, or 6 days after beetles were released into cages. Out of 30 plants of each cultivar, latex was collected from ten (five damaged and five undamaged checks, i.e., five replications per treatment) at each beetle exposure period (i.e., 1, 3, and 6 days after initiation of feeding damage). Latex (30 μl) was collected with a silanized glass microcapillary tube (Drummond Scientific Company, Broomall, PA, USA) from the leaf base (site of leaf lamina attachment to the stem and of rapid latex exudation upon cutting) of young and middle-aged leaves of individual plants immediately after cutting the tissue with a disposable scalpel blade (Feather, Osaka, Japan). Latex was applied, immediately after collection, onto the surface of a clear acetate plastic disc (2.5 cm diameter) with a microcapillary tube. A ring of felt (2.5 and 2.0 cm o.d. and i.d., respectively, and 3 mm thick) was placed on top of the latex-covered plastic disk. The nosecone of a color meter (ColorTec-PCM™, Clinton, NJ, USA) was placed on the felt ring to measure latex color. The color meter provides values for three parameters L^*, a^*, and b^*, specified by the Commission Internationale de 1'Esclairage. L^*, a^*, and b^* are three dimensions of the measured color, which provide a specific color value of the material; L^* represents the light–dark continuum with a range from 0 (black) to 100 (white); a^* represents the green–red continuum with a range from −60 (green) to +60 (red); and b^* represents the blue–yellow continuum with a range from −60 (blue) to +60 (yellow; Moss and Otten 1989). The data on latex L^*, a^*, and b^* were recorded at ten different time periods after collecting latex from a plant (0.5, 1, 2, 3, 5, 7, 9, 11, 13, and 15 min). The average of duplicate recordings was reported for each period.

The L^*, a^*, and b^* values were used to calculate two indices describing color characteristics of latex from damaged and undamaged plants: (1) whiteness index (WI; Eq. 1), as a measurement of whiteness, and (2) browning index (BI; Eq. 2), representing the purity of the brown color (Lohman and Hartel 1994; Maskan 2001).

$$\mathrm{WI} = 100 - \left[(100 - L^*)^2 + a^{*2} + b^{*2}\right]^{1/2} \quad (1)$$

$$\mathrm{BI} = \frac{[100(x - 0.31)]}{0.17} \quad (2)$$

where $x = \frac{a + 1.75L}{(5.645L + a - 3.012b)}$.

Collection of Latex for Feeding Bioassay and Enzyme Quantification One hundred and eighty plants of each cultivar were placed individually in cylindrical plastic cages (18.5 cm diameter×61.0 cm height) with three screened holes. Two male–female beetle pairs were placed into half (90 plants) of the cages of each cultivar; the other 90 plants of each cultivar were used as undamaged checks. Latex was collected from plants 1, 3, or 6 days after beetles were released into the cages. Out of the 180 plants of each cultivar, latex was collected from 60 (30 damaged and 30 undamaged checks) at each beetle exposure period (i.e., 1, 3, or 6 days after initiation of feeding damage). Out of each batch of 30 plants, latex from 15 was used for diet disk choice tests and latex from the other 15 plants was used to assess enzyme activity, as explained below. Each group of 15 plants was further divided into five groups (replicates) of three plants each for the collection of latex. An aliquot of 70 μl of latex was collected from each group of three plants for use in the assays described below. Females were weighed individually before releasing them on plants used for enzyme analysis and again either 1, 3, or 6 days after they were released into the cages, to determine weight change. The weight change was averaged over the six females per three plants as one replication of a total of five replications.

Choice-Test Bioassays with Latex from Damaged and Undamaged Plants Latex (70 μl) collected from plants as described above was applied immediately after collection to the top and side surfaces of a diet disk.. The experimental unit for the choice-test bioassay consisted of two diet disks: one treated with latex from beetle-damaged plants and the

other with latex from undamaged checks within each cultivar. In the control experimental units, two untreated diet disks were used. The diet disks were placed on the bottom of a plastic ventilated container (10×10×8 cm), and three male–female pairs of beetles were allowed to feed on the disks for 24 h at 25±1°C in a laboratory under a photoperiod of 14:10 h ratio of light to dark. The number of adults feeding on each diet disk was recorded 1, 2, 3, and 4 h after their release into the bioassay units. Dry weight of the diet consumed in 24 h was calculated as previously described (Sethi et al. 2008). Total diet consumed per three pairs of adults in 24 h was calculated by adding the consumption of the two diet disks in each replicate of each treatment.

Enzyme Activity Assays Activity of the enzymes PAL, PPO, and POX was assayed in the latices of Valmaine and Tall Guzmaine 1, 3, and 6 days after initiation of beetle damage. Collected latex was dispensed into −20°C chilled 1.5-ml microcentrifuge tubes on ice and immediately stored at −80°C until analyses.

Frozen latex (70 µl) was dissolved in 5 ml of 50 mM potassium phosphate buffer (pH 6.2) and centrifuged at 48,500×*g* for 45 min at 4°C (Model J2-HS, Beckman Instruments, Fullerton, CA, USA). The supernatant was collected and stored at −80°C until analyses. Total protein and enzyme activities were determined with a spectrophotometer (Model DU 640, Beckman Instruments, Fullerton, CA, USA). Total protein was estimated according to the modified Lowry's method (Peterson 1977) by using the Folin-Ciocalteau phenol reagent (Pierce Chemical, Rockford, IL, USA) and bovine serum albumin as a standard. Reaction mixture with boiled supernatant or without supernatant was used as control in all the enzyme assays.

PAL activity in latex was measured as described by Ke and Saltveit (1986) and Campos-Vargas and Saltveit (2002) with slight modifications. The supernatant was analyzed for PAL activity after addition of 200 µl of supernatant to 400 µl of 50 mM L-phenylalanine (dissolved in 20 mM potassium phosphate buffer, pH 8.8) and 400 µl of 50 mM potassium phosphate buffer (pH 8.8) and incubation at 40°C for 30 min. Absorbance was measured at 290 nm before and after incubation. PAL activity was expressed as the amount of PAL (µmol mg^{-1} h^{-1}) that produces 1 µmol of cinnamic acid in 1 h. Cinnamic acid (0–400 µmol in increments of 15 µmol) was used as a reference for quantification of PAL activity.

PPO activity was assayed following the methods of Sirinphanic and Kader (1985) and Loaiza-Velarde et al. (1997) with slight modifications. It was assessed by incubating 10 µl of supernatant with 500 µl of 1.6% catechol, 100 µl of 50 mM potassium phosphate buffer (pH 6.2), and 390 µl of distilled water. Absorbance of the mixture was read at 480 nm over a period of 2 min. One unit of PPO activity was defined as the amount of enzyme that produced an increase in absorbance of 0.1 per min at 480 nm. The linear portion of the curve was used to estimate the reaction rate. We did not add catalase to the reaction mixture prior to measurement for elimination of peroxidase-mediated phenolic oxidation. Therefore, our measured PPO activity may contain some residual peroxidase activity.

POX activity was determined by using the methods of Loaiza-Velarde et al. (1997) with slight modifications. It was determined by combining 10 µl of H_2O_2 (30%, *v*/*v*) in 50 µl of supernatant, 300 µl of 18 mM guaiacol, 100 µl of 50 mM potassium phosphate buffer (pH 6.2), and 540 µl of distilled water. Absorbance of the resulting mixture was examined at 420 nm over a period of 2 min. The POX activity (µmol mg $protein^{-1}$ min^{-1}) was determined by using guaiacol molar absorptivity (ε=26.6 M^{-1} cm^{-1}) at 420 nm. The linear portion of the curve was used to estimate the reaction rate.

Statistical Analysis The whiteness index of latex 0.5 min after collection was analyzed by using PROC GLM (SAS Institute 2003). The variables cultivar, latex type (from damaged or undamaged plants), and beetle exposure period (1, 3, or 6 days) were fixed effects, and replications were a random effect. Latex browning index was analyzed as a repeated measure by using multivariate analysis of variance (JMP release 7.0.1, SAS Institute Inc., Cary, NC, USA). The variables cultivar, latex type (from damaged or undamaged plants), and beetle exposure period (1, 3, or 6 days) were fixed. Five replications were randomly assigned to each level of latex and tested ten times (0.5, 1, 2, 3, 5, 7, 9, 11, 13, and 15 min after latex collection).

The numbers of insects feeding 1, 2, 3, or 4 h after release on diet disks treated with latex collected from plants with and without prior beetle exposure were analyzed as a repeated measures design by using PROC GLIMMIX. Separate analyses were run for disks from each cultivar at each beetle exposure period (1, 3, or 6 days). The variables latex type (from damaged or undamaged plants) and time period after beetle release (1, 2, 3, or 4 h) were fixed. Five groups of six beetles (i.e., replications) were randomly assigned to each level of latex and tested four times (1, 2, 3, and 4 h). Data on beetle fresh weight gain were analyzed by using PROC GLM with cultivar and beetle exposure period as fixed effects and replications as a random effect. Data on dry weight of diet consumed under choice tests were analyzed by using PROC GLM with latex and beetle exposure period as fixed effects. Replications were treated as a random effect for each cultivar.

Data on enzyme activities were analyzed by using PROC GLM with cultivar, latex type (damaged or undamaged), and beetle exposure period treated as fixed effects. Replications were treated as a random effect. Simple regression analysis was done to study the relationship between beetle fresh weight gain and enzymatic activities by using PROC GLM.

Tukey's honestly significant difference (HSD) test with a significance level of α=0.05 (SAS Institute 2003) was used for post hoc means separation.

Results

Color Characteristics of Latex from Damaged and Undamaged Plants The whiteness index of Valmaine latex was 1.83 times higher than that of Tall Guzmaine latex (26.20±1.01 vs. 14.49±0.92) when measured 0.5 min after collection ($F_{1,48}$= 101.60, P<0.001). It was lower in latex of damaged plants than in latex of undamaged plants (17.77±1.30 vs. 22.92± 1.44; $F_{1,48}$=19.70, P<0.001), but did not vary with length of beetle exposure period (1, 3, or 6 days; $F_{2,48}$=2.20, P= 0.122). None of the possible interactions among variables had a significant effect on the whiteness index.

Mean browning index of Valmaine latex (averaged over ten time periods after latex collection: 0.5, 1, 2, 3, 5, 7, 9, 11, 13, and 15 min) was 1.64 times higher than that of Tall Guzmaine latex ($F_{1,48}$=43.28, P<0.001; Fig. 1). It increased with beetle damage ($F_{1,48}$=23.29, P<0.001) and also with prolonged beetle exposure period (1, 3, or 6 days; $F_{2,48}$=9.91, P<0.001) and time period after latex collection (0.5, 1, 2, 3, 5, 7, 9, 11, 13, and 15 min; $F_{9,40}$=61.42, P< 0.001). Significant interaction was found between cultivar and treatment (damaged and undamaged; $F_{1,48}$=16.67, P< 0.001), but no interactions were found between cultivar and beetle exposure period ($F_{2,48}$=0.81, P=0.452), or between treatment and beetle exposure period ($F_{2,48}$=0.47, P= 0.631). Time period after latex collection interacted with beetle exposure period ($F_{9,41}$=2.62, P=0.0170), but not with cultivar ($F_{9,40}$=0.78, P=0.632) or treatment ($F_{9,40}$= 1.38, P=0.228).

Choice-Test Bioassays with Latex from Damaged and Undamaged Plants Similar mean numbers of beetles (averaged over four time periods after their release into the bioassay units—1, 2, 3, and 4 h) were counted on diet disks coated with latex from undamaged Valmaine plants and from plants that had been exposed to 1 day of feeding ($F_{1,8}$=2.08, P=0.159; Fig. 2a); however, latex collected from plants after 3 ($F_{1,8}$=18.96, P<0.001) and 6 days ($F_{1,8}$=14.43, P<0.001) of beetle exposure was significantly more deterrent than latex collected from undamaged plants. In Tall Guzmaine choice tests, similar mean numbers of beetles were counted on disks treated with latex collected from undamaged and damaged plants, regardless of the duration of beetle exposure (1 day: $F_{1,8}$= 0.075, P=0.785; 3 days: $F_{1,8}$=0.800, P=0.779; 6 days: $F_{1,8}$=0.047, P=0.830; Fig. 2b).

Adult *D. balteata* consumed less diet treated with latex from damaged Valmaine plants than diet treated with latex from undamaged plants ($F_{1,24}$=72.02, P<0.001; Table 1). There was a significant interaction between damage and beetle exposure period ($F_{2,24}$=3.67, P=0.041); beetles consumed less diet from disks treated with latex from plants that had been exposed to beetles for the longest time (6 days), whereas beetles consumed similar amounts of diet on the three sets of undamaged plants (Table 1). In the Tall Guzmaine choice test, beetle consumption of diet disks was similar, regardless of damage ($F_{1,24}$=0.216, P=0.646) or beetle exposure period ($F_{2,24}$=0.60, P=0.559; Table 1).

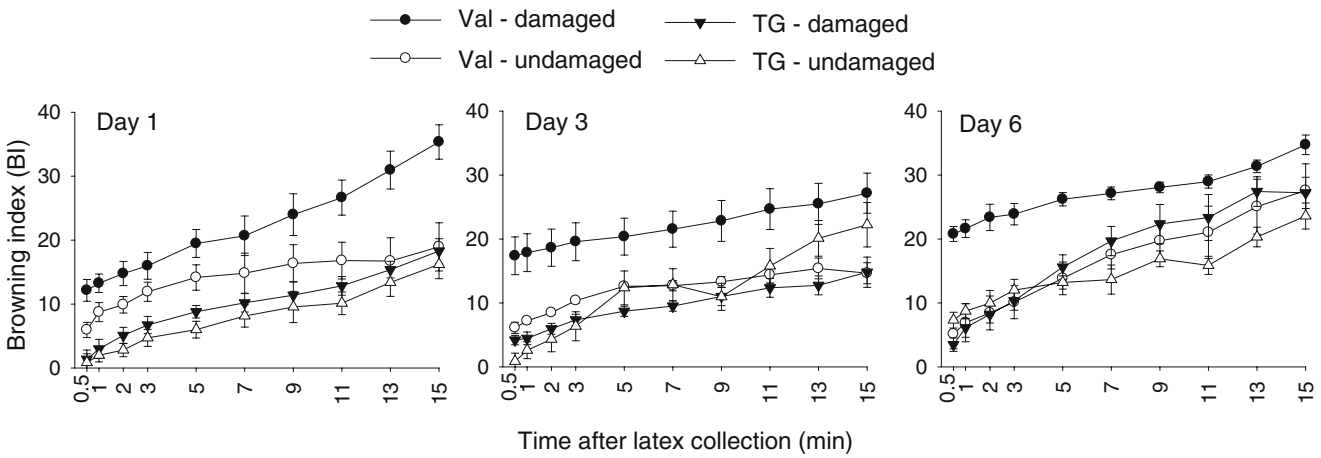

Fig. 1 Browning index (*BI*) of latex of two lettuce cultivars, Valmaine (*VAL*) and Tall Guzmaine (*TG*) 1, 3, and 6 days after initiation of feeding damage by adults of *D. balteata*. *Error bars* indicate standard error

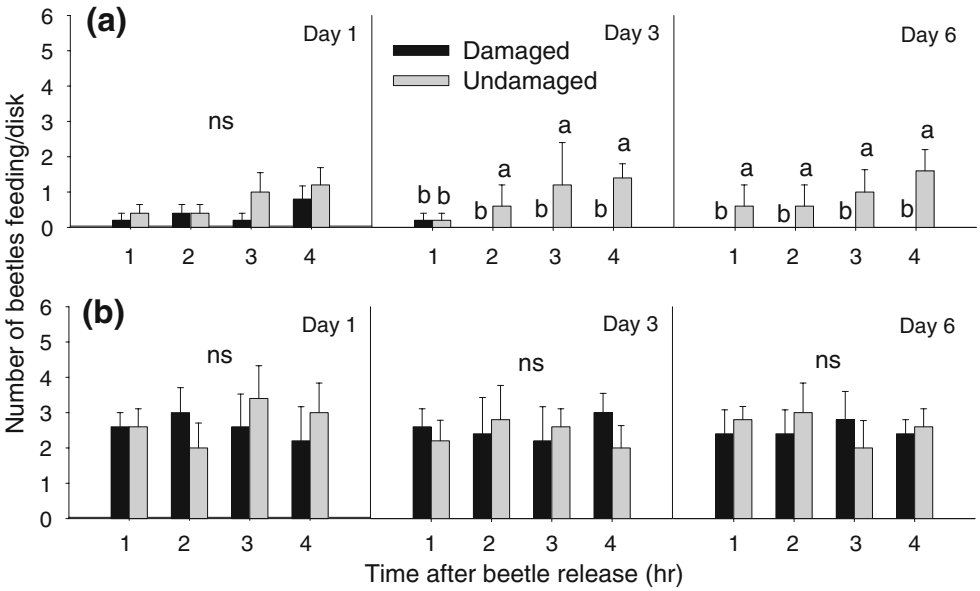

Fig. 2 Mean (+ standard error) number of adult *D. balteata* feeding on artificial diet disks when given a choice between disks treated with latex from damaged plants and disks treated with latex from undamaged plants of **a** Valmaine and **b** Tall Guzmaine 1, 2, 3, and 4 h after beetle release. *Bars capped with different letters within panel* (days 1, 3, or 6 within cultivar) differ significantly (Tukey's HSD test: $P \leq 0.05$)

Enzyme Activities and Total Protein Content Total protein content was 1.3-fold higher in Valmaine latex than in Tall Guzmaine latex ($F_{1,47}=91.77$, $P<0.001$; Fig. 3a). Interactions were found between cultivar and treatment (damaged or undamaged; $F_{1,47}=16.70$, $P<0.001$) and between cultivar and beetle exposure period ($F_{2,47}=7.61$, $P=0.001$). Total protein content in Valmaine after 6 days of feeding damage was 1.36-fold higher than after 1 day of feeding; however, there was no increase in protein content of Tall Guzmaine latex with increased duration of beetle feeding.

We did not find any enzymatic activity in either of the controls: no supernatant or boiled supernatant. In addition, the reaction mixture with boiled supernatant did not show any nonenzymatic browning. The specific activity of PAL was 3.44-fold higher in Valmaine latex than in Tall Guzmaine latex ($F_{1,47}=289.82$, $P<0.001$) and was influenced by treatment ($F_{1,47}=98.45$, $P<0.001$) and beetle exposure period ($F_{2,47}=7.96$, $P=0.001$; Fig. 3b). Interactions were found between cultivar and treatment ($F_{1,47}=20.96$, $P<0.001$) and between cultivar and beetle exposure period ($F_{2,47}=7.36$, $P=0.002$). PAL activity in Valmaine latex was significantly increased after 3 (1.81-fold) and 6 days (1.54-fold) of feeding damage, relative to the 1-day beetle exposure period. No increase in PAL activity was observed over time in the latex of Tall Guzmaine.

The specific activity of PPO was 4.37-fold higher in Valmaine latex than in Tall Guzmaine latex ($F_{1,47}=358.32$, $P<0.001$) and was affected by treatment ($F_{1,47}=80.31$, $P<0.001$) and beetle exposure period ($F_{2,47}=8.25$, $P<0.001$; Fig. 3c). Interactions were found between cultivar and treatment ($F_{1,47}=74.86$, $P<0.001$) and between cultivar and beetle exposure period ($F_{2,47}=11.65$, $P=0.002$). PPO activity in Valmaine latex was increased significantly over the 1-day activity 3 (1.74-fold) and 6 days (1.78-fold) after feeding initiation, but not in Tall Guzmaine latex.

The specific activity of POX was 2.1-fold higher in Valmaine latex than in Tall Guzmaine latex ($F_{1,47}=35.49$, $P<0.001$; Fig. 3d) and was affected by treatment ($F_{1,47}=39.29$, $P<0.001$) and beetle exposure period ($F_{2,47}=4.92$, $P=0.011$). Interactions were found between cultivar and

Table 1 Consumption of artificial diet by six *D. balteata* adults after 24 h in choice tests

Days of beetle exposure	Diet consumption (mg)[a]			
	Valmaine		Tall Guzmaine	
	Damaged	Undamaged[b]	Damaged	Undamaged[b]
1	5.7±1.1b	9.5±1.2a	19.3±1.9a	17.5±1.4a
3	3.1±0.6bc	10.4±0.7a	18.5±1.9a	16.9±2.4a
6	1.7±0.4c	10.7±1.4a	13.0±2.3a	19.0±3.0a

Each beetle was presented two diet disks: one treated with latex from plants damaged by exposure to beetles for different periods of time and one treated with latex from undamaged plants. Tests with latex from Valmaine and Tall Guzmaine were conducted and analyzed separately

[a] Mean ± standard error. Each table entry followed by different letters within a column (=cultivar-Valmaine or Tall Guzmaine) differed significantly ($P \leq 0.05$) by ANOVA and Tukey's HSD test

[b] Each beetle exposure period had separate check groups of undamaged plants

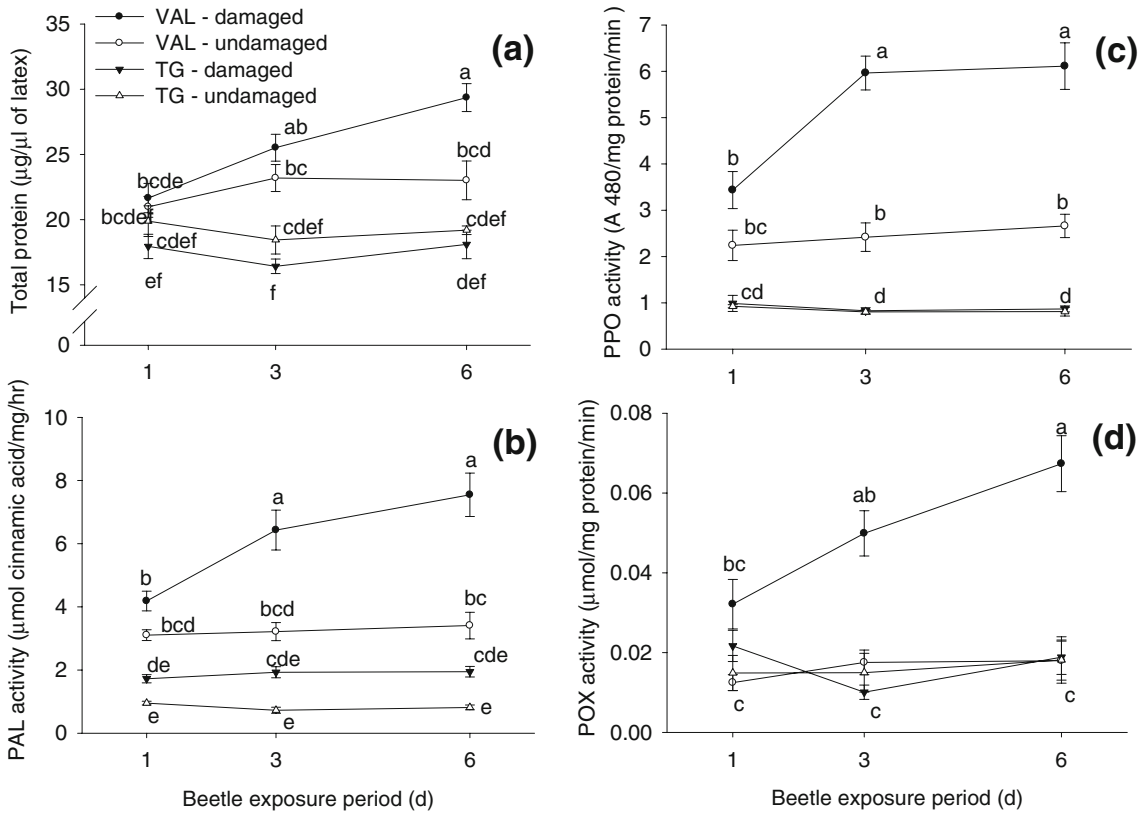

Fig. 3 **a** Total protein content and activities of three enzymes, **b** phenylalanine ammonia lyase (*PAL*), **c** polyphenol oxidase (*PPO*), and **d** peroxidase (*POX*) in two lettuce cultivars, Valmaine (*VAL*) and Tall Guzmaine (*TG*) 1, 3, and 6 days after initiation of feeding damage by adult *D. balteata*. *Error bars* indicate standard error. *Points capped with different letters within a panel* differ significantly (Tukey's HSD test: $P \leq 0.05$)

treatment ($F_{1,47}$=35.45, P<0.001) and between cultivar and beetle exposure period ($F_{2,47}$=5.16, P=0.009). POX activity in Valmaine latex was significantly increased over the 1-day activity 3 (1.56-fold) and 6 days (2.1-fold) after feeding initiation but not in Tall Guzmaine latex.

Relationship Between Female Weight Gain and Enzyme Activity Female beetles confined on Valmaine plants gained less weight than females confined on Tall Guzmaine ($F_{1,23}$=1269.92, P<0.001). Female fresh weight gain was affected by beetle exposure period ($F_{2,23}$=30.42, P<0.001) and the interaction between cultivar and beetle exposure period ($F_{2,23}$=161.35, P<0.001). Beetles caged on Tall Guzmaine for 1 day gained twice as much weight as those confined on Valmaine (0.84±0.03 mg vs. 0.38± 0.04 mg), 12 times as much when caged for 3 days (1.21± 0.03 mg vs. 0.11±0.02 mg) and 51 times as much when confined for 6 days (1.83±0.07 mg vs. −0.04±0.02 mg). Beetles lost weight the longer they were confined on Valmaine. Furthermore, significant negative linear relationships were found between female fresh weight gain and activities of each enzyme (PAL, PPO, and POX) in latex from damaged plants of Valmaine (Fig. 4). No such relationship was found between female fresh weight gain and any of the enzyme activities in latex from Tall Guzmaine.

Discussion

Resistance to *D. balteata* adults was increased in Valmaine compared to Tall Guzmaine after 24 h of feeding damage. Latex from damaged Valmaine plants was more deterrent to beetle feeding than latex from undamaged plants, whereas damage did not affect deterrence of latex from Tall Guzmaine plants. Earlier tests by Huang et al. (2003) detected localized induced resistance but no systemic resistance in Valmaine after 2 days of feeding damage by *D. balteata*. Differences in methodology between their study and ours may explain the differing results. In their study, damage was inflicted by confining a pair of beetles on an intact leaf and testing the latex from that leaf and the one directly above it for feeding deterrence. In our system, damage was inflicted by allowing two pairs of beetles to feed anywhere on a whole plant. So in our study, beetles might have damaged several leaves and consequently the plant might have responded collectively to a greater extent

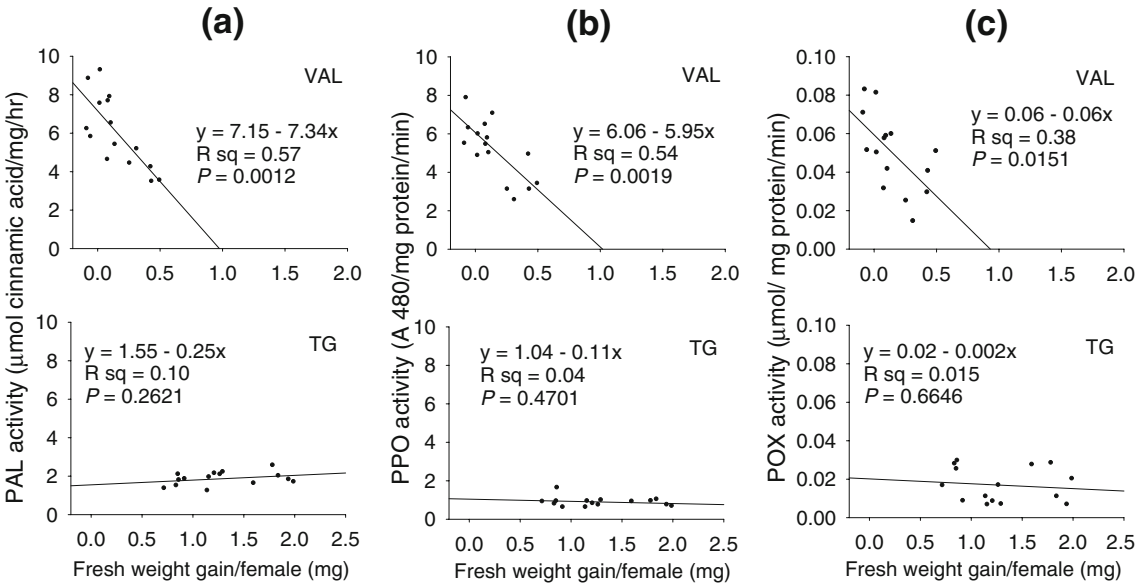

Fig. 4 Relationship between fresh weight gained by female *D. balteata* feeding on two lettuce cultivars, Valmaine (*VAL*) and Tall Guzmaine (*TG*) and activity of **a** PAL, **b** PPO, and **c** POX enzymes in latex collected from plants after 1, 3, and 6 days of exposure to beetle feeding damage

to induce systemic resistance. In addition, we used latex that was collected at several locations within the plant, thus representing three developmental ages of leaves, i.e., young, middle, and mature. The change in palatability of Valmaine latex may be due to a change in the concentration of its constituents. Malcolm and Zalucki (1996) found increased level of resistance in milkweed, *Asclepias syriaca* L. (Asclepiadaceae), 24 h after damage by monarch butterfly larvae, *Danaus plexippus* L. (Lepidoptera: Nymphalidae: Danainae), due to increase in the levels of cardenolides.

The amount of total protein increased in latex from Valmaine after beetle damage, but it did not change in Tall Guzmaine. Ni et al. (2001) also found a significant increase in the total protein content in wheat cultivars after damage by the Russian wheat aphid. Laticifer proteins from rooster tree (Asclepiadacae) greatly reduced survival and development of larval instars of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann; Diptera: Tephritidae), and velvetbean caterpillar, *Anticarsia gemmatalis* (Hübner; Lepidoptera: Noctuidae), when mixed in artificial diet (Ramos et al. 2007).

Latex from Valmaine was whiter than latex from Tall Guzmaine. Such differences in whiteness could indicate differences in concentrations of defensive secondary metabolites (terpenoids, alkaloids, tannins, proteins, and sterols; Esau 1965). The quantity of latex exuded by Tall Guzmaine plants decreased with increasing duration of beetle exposure, and latex collected after 3 and 6 days of feeding damage was also less viscous and more watery and translucent, whereas the consistency of Valmaine latex remained constant after damage (Sethi 2007). Latex from damaged plants was less white than that from undamaged plants, probably due to increased browning. The intensity of browning in Valmaine latex was higher in both undamaged and damaged plants compared to Tall Guzmaine plants. The latex from Valmaine plants fed upon by beetles for 3 or 6 days browned to a deeper degree than did latex collected after 1 day of feeding damage. However, the intensity of browning remained the same in Tall Guzmaine latex after beetle damage. Upon wounding, latex turns brown over time due to the production of quinones that are catalyzed by PPO (Wang and Constabel 2004). Browning intensity has been associated with higher resistance to pathogens; browning was much more intense in a disease-resistant clone of rubber tree than in a susceptible clone (Wititsuwannakul et al. 2002). Valentines et al. (2005) also found a positive relationship between browning potential and disease resistance in apples. Enhanced browning of callus and pericarp in a resistant corn line has been associated with increase in resistance against the corn earworm, *Helicoverpa zea* (Boddie; Lepidoptera: Noctuidae), and the dusky sap beetle, *Carpophilus lugubris* Murray (Coleoptera: Nitidulidae), compared to a susceptible line (Dowd 1994; Dowd and Norton 1995).

The production of phenylpropanoid compounds plays an important role in plant defense (Hahlbrock and Scheel 1989). Phenylalanine ammonia lyase is the first committed enzyme in the phenylpropanoid pathway (Dixon and Paiva 1995). Its de novo synthesis and increased activity is an initial response to mechanical wounding (Campos-Vargas and

Saltveit 2002) that ultimately results in increased concentrations of phenolic compounds in lettuce (Loaiza-Velarde et al. 1997). The phenylpropanoid pathway starts with the deamination of phenylalanine to cinnamic acid due to the action of PAL. The enhanced activity of PAL results in an increased production and accumulation of several phenolic compounds that are sequestered in the vacuole. These compounds can be oxidized to strong electrophilic quinones (brown substances) by PPO when membranes become disrupted. In addition, wounding also results in an increased expression of POX and lignin formation (Ribereau-Gayon 1972). The activities of all three oxidative enzymes were increased significantly in Valmaine latex after 3 and 6 days of *D. balteata* feeding damage, but they remained constant in Tall Guzmaine latex. Even the constitutive levels of PAL and PPO in latex from undamaged Valmaine plants were significantly higher than levels in latex from Tall Guzmaine. In addition, enzyme activities are even higher in Valmaine when expressed on a volume basis because the amount of total protein is higher in Valmaine than Tall Guzmaine. The increased intensity of browning in Valmaine latex observed in our study may be due to higher activity of PPO. Alteration in the levels of these enzymes due to insect feeding has been documented by many other researchers (Felton et al. 1994; Stout et al. 1999; Chaman et al. 2001; Ni et al. 2001; Wang and Constabel 2004).

Increases in insect resistance due to higher activity of PPO in potato, cotton, soyabean, tomato, rubber tree, poplar, and barley (Gregory and Tingey 1981; Hedin et al. 1983; Duffey and Felton 1991; Felton et al. 1994; Chaman et al. 2001; Wititsuwannakul et al. 2002; Wang and Constabel 2004) and POX in tomato (Stout et al. 1999) and barley (Chaman et al. 2001) have been correlated with decreases in insect fitness. Higher activity of PAL was found in resistant cultivars of lettuce infested with lettuce root aphid, *Pemphigus bursarius* (L.; Cole 1984) and barley infested with greenbug, *Schizaphis graminum* (Rondani; Chaman et al. 2003). The activity of PAL was also increased in strawberry leaves as a result of infestation by two-spotted spider mite, *Tetranychus urticae* (Inoue et al. 1985). We found a strong negative relationship between female beetle weight gain and activities of PAL, PPO, and POX, indicating a correlation between increased enzymatic activities and decreased beetle fitness. Female beetles confined for 1 day on Valmaine plants had gained weight, suggesting that at least 2 days of feeding are necessary to induce resistance (as shown by Huang et al. 2003). Beetles were observed tunneling and presumably feeding in the midrib tissue near the proximal end of the leaf. However, after 3 days, beetles were not observed feeding and they lost weight over the remaining 3 days of the experiment.

Based on our results, we hypothesize that increased levels of PAL, PPO, and POX in Valmaine after *D. balteata* damage result in increased production of defensive secondary metabolites. Solvent extraction of deterrent compounds from latex of undamaged Valmaine plants also suggests the presence of biologically active compounds that are constitutively expressed (Sethi 2007). Therefore, it appears that Valmaine possesses both constitutive and induced resistance mechanisms, mediated through latex, which act together to enhance its resistance against *D. balteata*. Further research is required to characterize both constitutive and insect damage-induced gene expression to understand the resistance mechanism in Valmaine against multiple insects and ultimately to support breeding programs for the development of resistant cultivars with superior horticultural traits.

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Vicia faba–Lygus rugulipennis Interactions: Induced Plant Volatiles and Sex Pheromone Enhancement

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Abstract The profiles of volatile chemicals emitted by *Vicia faba* plants damaged by *Lygus rugulipennis* feeding, and by feeding plus oviposition, were shown to be quantitatively different from those released by undamaged plants. Samples of volatile chemicals collected from healthy plants, plants damaged by males as a consequence of feeding, plants damaged by females as a consequence of feeding and oviposition, plants damaged by feeding with mated males still present, and plants damaged by feeding and oviposition with gravid females still present, showed significant differences in the emission of hexyl acetate, (*Z*)-β-ocimene, (*E*)-β-ocimene, (*E*)-β-caryophyllene, and methyl salicylate. In particular, treatments with mated females present on plants had a significant increase in emission levels of the above compounds, possibly due to eggs laid within plant tissues or active feeding, compared with undamaged plants and plants damaged by males feeding, with or without insects still present. Furthermore, the pheromonal blend released by mated *L. rugulipennis* females, mainly comprising hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal, was enhanced when females were active on broad bean plants, whereas such an increase was not observed in males. Both sexes gave electroantennogram responses to green leaf volatiles from undamaged plants and to methyl salicylate and (*E*)-β-caryophyllene emitted by *Lygus*-damaged plants, suggesting that these compounds may be involved in colonization of host plants by *L. rugulipennis*. In addition, mated males and females were responsive to hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal released by mated females on *V. faba*, indicating that these substances could have a dual function as a possible aggregation pheromone in female–female communication, and as a sex pheromone in female–male communication.

Keywords *Lygus rugulipennis* · Miridae · Heteroptera · *Vicia faba* · Host plant volatiles · Induced volatiles · Sex pheromone · Aggregation pheromone

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Introduction

Natural "green plant odor" is a blend that comprises up to several hundred volatile compounds (Visser 1986; Price 1997), many of which are used by herbivorous insects to recognize host plants from a distance (Bernays and Chapman 1994). Plants frequently are damaged by insects in the natural habitat, and several studies have demonstrated that such damage can trigger production of volatile chemicals different from those released by undamaged or mechanically damaged plants (e.g., De Moraes et al. 1998; Turlings et al. 1998; Dicke and van Loon 2000; Kessler and Baldwin 2001).

Much of the knowledge of plant responses to herbivores has been acquired from studies on leaf-chewing insects that damage foliage extensively (e.g., De Moraes et al. 1998; Dicke and van Loon 2000; Kessler and Baldwin 2001); less is known about plant responses to non-chewing or sucking insects (Du et al. 1998, Rodriguez Saona et al. 2002; Colazza et al. 2004b). Only a few studies have focused on

induction of plant volatiles by insect oviposition and the consequent attraction of egg parasitoids (e.g., Meiners and Hilker 2000; Wegener et al. 2001; Colazza et al. 2004a, b). The profile of volatile chemicals emitted by herbivorous insects may be altered during oviposition and/or feeding on host plants, compared with the chemicals released in the absence of plants (Deng et al. 2004). Furthermore, plant stimuli can synergize the efficacy of external compounds, such as insect pheromones, and also influence herbivore sexual communication by enhancing pheromone production and release (e.g., Landolt et al. 1994; Deng et al. 2004; Yang et al. 2003).

The insect used in this study is the European tarnished plant bug, *Lygus rugulipennis* Poppius (Heteroptera: Miridae). It is the most common species of the genus *Lygus* in Europe, where it is widely distributed on both herbaceous and arboreous wild and cultivated plants, with records on more than 320 host plants (Holopainen and Varis 1991). This bug damages plants either by feeding, through injection of saliva rich in degrading enzymes, or by incisions made for oviposition with eggs embedded within the plant tissue. In the *Vicia faba–L. rugulipennis* system, it has been observed in the wind tunnel that both sexes move toward damaged host plants, and that the presence of mated conspecifics on plants enhances the responses of females, whereas the presence of conspecific eggs reduces the responses of both sexes (Frati et al. 2008). In addition, both males and females are less responsive to conspecifics alone compared to the host plant-conspecific complex (Frati et al. 2008). Virgin female *Lygus* bugs release a sex pheromone consisting of hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal (Ho and Millar 2002; Innocenzi et al. 2004, 2005).

Damage by the American species, *Lygus hesperus* Knight, that feeds on cotton, maize, and alfalfa plants, induces an increase in constitutive volatile compounds and the ex novo production of other molecules (Rodriguez-Saona et al. 2002; Blackmer et al. 2004). Induction of plant volatiles also has been shown by other non-chewing insects such as *Trialeurodes vaporariorum* (Homoptera: Aleyrodidae; Birkett et al. 2003) on *Phaseolus vulgaris*, *Nezara viridula* (Heteroptera: Pentatomidae; Colazza et al. 2004a), and *Acyrthosiphon pisum* (Harris) (Homoptera: Aphididae) on broad bean plants (Du et al. 1998), and *Murgantia histrionica* (Heteroptera: Pentatomidae) on cabbage plants (Conti et al. 2008).

The main goal of the present work was to determine whether or not oviposition and/or feeding damage by *L. rugulipennis* on the host plant, with or without the presence of conspecifics, alters the volatile chemical profile of broad bean (*V. faba* L.), and whether the sex pheromone blend is enhanced by the presence of the host plant. A second objective was to evaluate the electrophysiological responses of *L. rugulipennis* to volatile chemicals emitted by healthy and damaged plants, with and without conspecifics.

Methods and Materials

Insect Rearing *L. rugulipennis* were reared inside plastic cages (18×10.5×10 cm) containing strips of paper to increase the walking-surface area, located in a growth chamber (20±1°C, 50–60% RH, and photoperiod L12: D12). Adults were reared on fresh green beans (*P. vulgaris* L.) with food replaced three times a week. Beans containing eggs were transferred into other cages for nymph development. Upon hatching, nymphs also were fed on fresh green beans until adults emerged.

Mated *L. rugulipennis* females and males of the same age (~12–14 days old) were used in the experiments, as in behavioral assays previously carried out (Frati et al. 2008). It was assumed that insects were mated, because males and females were kept together after emergence. To reduce behavioral variability, each individual was isolated, 15–16 h before an experiment, inside a plastic pot (4 cm diam.×6.5 cm high), and allowed to feed on fresh green beans under the same conditions as the experiment.

Plants Broad bean plants, *V. faba* L. (cv. Sutton), were grown from seeds sown in plastic pots (8×8×10 cm) at a density of 1 plant/pot, using a compost mix (prepared for Rothamsted Research by Petersfield Products), and kept in a glasshouse at 20°C, photoperiod 16L:8D and 50–60% RH. In all air entrainments, only plants with four to five fully expanded true leaves were used. Plants were transferred, 2–3 h before the headspace volatile collections, into a climate-controlled chamber (20°C, 60±5% RH, and photoperiod 16L:8D).

Headspace Volatile Collection The following headspace volatile collections were carried out: (1) healthy plant as control; (2) plant damaged by feeding males with males removed; (3) plant damaged by feeding and oviposition of females with females removed; (4) plant damaged by males feeding with males present; (5) plant damaged by oviposition and feeding of females with females present; 6) mated males; and (7) gravid females. Plants damaged by *L. rugulipennis* were obtained by placing six mated adults on each plant (20°C, 60±5% RH, and photoperiod 16L:8D) inside a fine plastic mesh, and leaving the insects to feed (males) or feed and oviposit (females). After 24 h, insects were removed or left on the plant (as appropriate), and headspace collections were carried out. To verify oviposition on plants, numbers of eggs were counted after collection of volatiles, and only data from plants with ten or more eggs were used in the analysis.

In entrainments with mated adults alone, insects were placed inside a glass chamber (with an inlet and an outlet) that contained a plastic cup with moist cotton wool and no food (Frati et al. 2008). In plant entrainments, two halves of a cylindrical glass chamber (13 cm i.d.×22 cm h), with an inlet and an outlet, were placed around part of the plant, with the stem or petiole passing through a small hole in the central flange. Before use, the chamber was washed with water and detergent, wiped with acetone, and kept in an oven overnight at ~180°C. Approximately 30 min before collection started, plants, plants plus insects, or insects alone, were transferred into the chambers. Air, purified by passage through an activated charcoal filter, was pumped at a flow of ~1,300 ml min^{-1} and divided between two chambers. The air was simultaneously drawn from each chamber at 500 ml min^{-1} through a glass tube containing Porapak Q (60 mg, 80–100 mesh) to trap plant and/or insect volatiles. The Porapak was conditioned by washing with redistilled diethyl ether, and heating (132°C) overnight in a nitrogen stream (100 ml min^{-1}). Entrainments were performed in a temperature-controlled room with overhead fluorescent lights providing long-day conditions (lights on from 6:00 h to 22:00 h). Each volatile collection was repeated seven times (eight in the case of collections from mated females), beginning between 11.00–12.00 h and ending 24 h later. Traps were extracted with 700 μl of distilled diethyl ether, and extracts stored at −20°C in glass vials with Teflon cap-liners until used for analysis. Before analysis, each sample was concentrated in a nitrogen flow to approximately 200 μl.

Gas Chromatography Extracts were analyzed on a Hewlett-Packard 6890 gas chromatograph, equipped with a cool-on-column injector (30°C), a flame ionization detector (FID), and a 50 m×0.32 mm i.d. HP-1 capillary column, and hydrogen as carrier gas. The oven temperature was maintained at 30°C for 2 min and then increased at 10°C min^{-1} to 250°C. For *Lygus* volatiles, it was not possible to separate some peaks with the HP-1 column, so these extracts were further analyzed on an Agilent 6980N gas chromatograph, equipped with a cool-on-column injector (30°C), FID, and a 30 m×0.32 mm i.d. DB-Wax column, and hydrogen as carrier gas. The oven temperature was maintained at 30°C for 2 min and then increased at 10°C min^{-1} to 230°C.

Compounds were qualitatively identified, initially by comparing retention indices with a database of values for common plant volatiles, and subsequently using both gas chromatography–mass spectrometry (GC–MS) and co-injection of synthetic standards from commercial sources by using two columns of different polarity (Pickett 1990). The amount of each compound (nanogram) was estimated by comparison of peak area with that of an internal standard (dodecane, 100 ng). As different compounds may have different responses in terms of GC peak areas, the response factor for each identified compound was calculated from the area of a known amount of authentic compound.

GC–MS analyses were carried out by using a Thermo-Finnigan MAT95XP. GC columns (HP-1 and DB-wax), and conditions were similar to those described above except a temperature program of 30°C (held for 5 min) to 250°C at 5°C $min^{-1}$was used, and the carrier gas was helium.

Statistical Analysis Data were analyzed by one-way ANOVA (Statistica 6.0, Statsoft Inc. 2001). Before analysis, data were log-transformed to satisfy ANOVA assumptions (Zar 1999).

Chemicals (*E*)-2-hexenyl butanoate was synthesized from commercially available (*E*)-2-hexenoic acid by conversion to the methyl ester and reduction with diisobutylaluminum hydride to the corresponding alcohol. Reaction of (*E*)-2-hexenol with butanoyl chloride in the presence of 4-dimethylaminopyridine and triethylamine gave the desired ester in good yield after purification by flash chromatography. 4-Oxo-(*E*)-2-hexenal was prepared from 2-ethylfuran, using aqueous *N*-bromosuccinimide to promote oxidative ring opening (Moreira and Millar 2005). (*E*)-β-Ocimene was prepared using a three-step synthesis in which 3-methyl-2,5-dihydro-thiophene 1,1-dioxide, obtained by condensation of isoprene with sulfur dioxide in a sealed steel reactor, was deprotonated at low temperature in THF using sodium bis(trimethylsilyl)amide and then treated with excess 1-bromo-3-methyl-but-2-ene. The resulting 3-methyl-2-(3-methyl-but-2-enyl)-2,5-dihydro-thiophene 1,1-dioxide was treated with lithium aluminum hydride in refluxing diethyl ether to yield the (*E*)-β-ocimene in good yield and high purity (Gaoni 1977).

Authentic samples of (+) and (−)-germacrene-D were obtained by incubation of farnesyl pyrophosphate with purified, expressed (+) or (−)-germacrene-D synthase and subsequent hexane extraction and purification through a short column of silica gel (BDH, 40–63 μm)/$MgSO_4$ (10:1) (Prosser et al. 2004). (*E*,*E*)-α-Farnesene had been synthesized earlier at Rothamsted Research, as described by Cook et al. (2007), and was available to us. All other chemicals were obtained commercially (hexyl acetate: Aldrich; 1-hexanol: Sigma; linalool: Fluka; (*E*)-β-caryophyllene: Pfaltz & Bauer; (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate: Alfa Aesar; indole, methyl salicylate: Avocado Research Chemical; (*Z*)-ocimene: Bush-Boake Allen, London).

Electrophysiology Electroantennogram (EAG) recordings were made using Ag–AgCl glass electrodes filled with saline solution (as in Maddrell 1969, but without glucose). The insect head was excised, placed within the indifferent electrode, and the terminal process of the antenna, with the

Table 1 Mean amount ± SE of volatiles collected from healthy *Vicia faba* plants, plants damaged by *Lygus rugulipennis* males that had been removed, plants damaged by females that had been removed, plants damaged by males still present, plants damaged by females still present

Treatment	*N*	Compounds (ng/h)[a]					
		Alcohols		Alkaloids	Esters		
		(Z)-3-Hexenol	1-Hexanol	Indole	(Z)-3-hexenyl acetate	Hexyl acetate	Methyl salicylate
Healthy	7	194.86±40.87a	2.69±0.70a	1.82±0.44a	55.28±16.42a	0.36±0.20b	0.44±0.04c
Damaged by ♂	7	224.07±65.14a	2.37±0.57a	2.29±0.58a	47.13±10.04a	0.09±0.06b	2.00±0.54bc
Damaged + ♀	7	196.80±48.34a	1.96±0.41a	2.27±0.48a	52.11±9.94a	0.20±0.15ab	2.86±0.65ab
Damaged + ♂	7	193.29±34.98a	2.04±0.33a	3.51±1.33a	41.47±9.90a	0.42±0.24ab	2.40±0.58b
Damaged + ♀	7	94.90±34.21a	1.82±0.50a	2.95±1.13a	81.55±46.48a	1.41±0.41a	8.90±2.98a

Total duration of volatile collection was 24 h, starting at ~11.00–12.00 AM

[a]The mean amount of each compound (ng/h) was estimated by comparison of peak area with that of an internal standard (dodecane, 100 ng). Numbers in column followed by the same letter indicate means that are not significantly different ($P>0.05$, ANOVA).

N number of replicates, *TMTT* (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene

tip having removed to ensure good contact, introduced into the recording electrode,. The signals were passed through a high impedance amplifier (UN-06, Syntech, The Netherlands) and analyzed with a customized software package (Syntech). The stimulus was delivered into a purified airstream (1 l min^{-1}) flowing over the preparation.

The coupled GC–EAG system used has been described previously (Wadhams 1990). An AI 93 GC equipped with a cold on-column injector and a FID was used. The column was a 30 m×0.32 mm i.d. HP-WAX column, the temperature program was 40°C (held for 1 min) to 220°C at 10°C min^{-1}, and the carrier gas was hydrogen. The outputs from the EAG amplifier and the FID were monitored simultaneously and analyzed with the Syntech software package. Solutions (2 µl), of volatiles from healthy plants, from feeding and oviposition-damaged plants without females ($N=4$ for female; $N=6$ for male), and from feeding and oviposition-damaged plants with gravid females ($N=4$), were analyzed using antennal preparations of mated males and females. A peak on the GC was determined to be electrophysiologically active if it elicited a response on three or more preparations.

Results

GC analysis of the volatile chemicals trapped on Porapak Q showed significant differences in total amounts released by the different treatments of *V. faba* plants (Table 1 and Fig. 1). Probably, the biggest difference was that between *V. faba* plants with females on, and the other treatments. Among the green leaf alcohols and esters, more hexyl acetate was emitted by plants with females present (i.e., damaged by feeding and oviposition) than by undamaged plants (Table 1 and Fig. 1; $F=5.309$; $df=4, 30$; $P=0.002$). However, in the case of (*Z*)-3-hexenol ($F=2.117$; $df=4, 30$; $P=0.103$), 1-hexanol ($F=0.255$; $df=4, 30$; $P=0.905$), and (*Z*)-3-hexenyl acetate ($F=0.186$; $df=4, 30$; $P=0.944$), no significant differences were detected among treatments.

With regard to the shikimic acid pathway products, the amount of methyl salicylate released by plants on which females were present was greater than all other treatments, except that on which females had been present (Table 1 and Fig. 1). Moreover, plants on which females were or had been present, or on plants on which males were present, released significantly greater amounts of this chemical than undamaged plants. In the case of indole, there were no

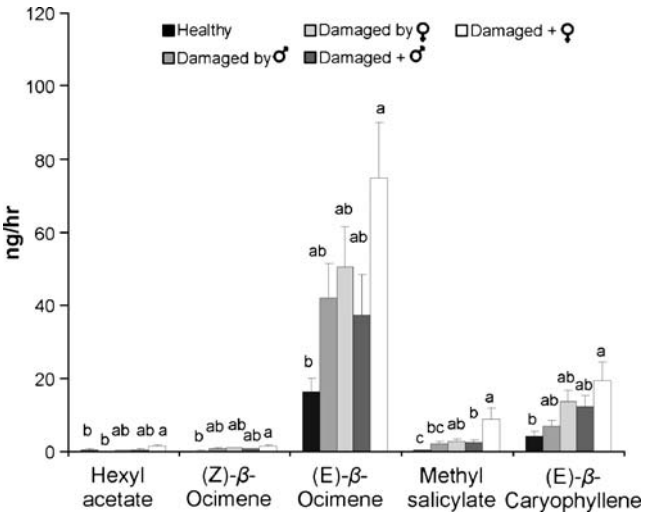

Fig. 1 Release rates of the major volatile compounds collected from *Vicia faba* plants with different treatments, consisting of damage by *Lygus rugulipennis*: undamaged plants, plants damaged by males (feeding) after which males were removed, plants damaged by females (feeding and oviposition) after which females were removed, plants damaged by males that were still present, plants damaged by females that were still present. *Each bar* represents a mean ± SE. *Columns with the same letter* indicate means that are not significantly different ($P>0.05$, ANOVA)

Terpenoids						
Linalool	(*E*)-β-caryophyllene	(*Z*)-β-ocimene	(*E*)-β-ocimene	Germacrene D	(*E*, *E*)-α-farnesene	TMTT
0.70±0.17a	4.20±1.21b	0.16±0.11b	16.32±3.75b	0.48±0.16a	1.21±0.25a	15.43±2.96a
1.72±0.44a	6.61±1.68ab	0.64±0.24ab	41.94±9.46ab	1.19±0.34a	1.78±0.26a	13.88±3.40a
1.36±0.23a	13.44±3.03ab	0.90±0.25ab	50.30±11.34ab	1.66±0.88a	1.89±0.38a	15.95±3.27a
0.96±0.32a	12.06±3.09ab	0.60±0.24ab	37.16±11.31ab	1.10±0.49a	2.56±0.69a	20.51±7.34a
1.93±0.65a	19.12±5.31a	1.40±0.27a	74.75±15.22a	1.77±0.38a	3.57±1.08a	19.23±3.82a

significant differences (F=0.142; df=4, 30; P=0.965; Table 1) among the five treatments.

The monoterpenes (*Z*)-β-ocimene (F=3.967; df=4, 30; P=0.011) and (*E*)-β-ocimene (F=2.600; df=4, 30; P=0.040) were released in greater quantities from plants on which females were present than on undamaged plants (Table 1 and Fig. 1). A similar result (i.e., quantity released by plants with females was greater than that released by control plants) was observed for the sesquiterpene (*E*)-β-caryophyllene (Table 1 and Fig. 1; F=3.581; df=4, 30; P=0.017). In the case of linalool (F=1.520; df=4, 30; P=0.222), germacrene D (F=1.364; df=4, 30; P=0.270), (*E,E*)-α-farnesene (F=1.666; df=4, 30; P=0.184), and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (F=1.170; df=4, 30; P=0.952), there were no significant differences among treatments (Table 1).

The three main components of the sex pheromone of *L. rugulipennis*, hexyl butyrate (F=9.480; df=3, 25; P<0.001), (*E*)-2-hexenyl butyrate (F=13.627; df=3, 25; P<0.001), and (*E*)-4-oxo-2-hexenal (F=20.371; df=3, 25; P<0.001), were emitted in larger quantities by females when they were on broad beans than when they were not, whereas no such increase was observed for males (Fig. 2). Overall, females on plants released larger amounts of these three compounds than any of the other treatments (Fig. 2). In addition, when no plant was present, the amount of (*E*)-4-oxo-2-hexenal released by females was greater than that released by males (Fig. 2).

GC–EAG analysis of volatiles from undamaged plants showed that the green-leaf volatiles (*Z*)-3-hexenol, 1-hexanol, and (*Z*)-3-hexenyl acetate triggered responses in both mated females and males (Fig. 3a). When the volatiles from a female-damaged plant from which the females had been removed were tested, (*E*)-β-caryophyllene and methyl salicylate elicited EAG responses in both sexes (Fig. 3b). In addition, (*Z*)-3-hexenyl acetate elicited female and male responses in one preparation, while 1-hexanol and (*Z*)-3-hexenol triggered female and male responses in two preparations, respectively. Finally, when the volatiles from a female-damaged plant with females present were tested, only antennal responses to the three main sex pheromone components were recorded in both mated males and females (Fig. 3c).

Discussion

L. rugulipennis, during feeding and/or oviposition on broad bean plants, induces changes in the emission of several volatile compounds as compared to undamaged plants. The most profound changes were observed when mated females were present, and to a lesser extent when females had been, on the plant. The difference in the amount of volatile emissions is due possibly to endophytic oviposition by *Lygus* causing laceration of tissues, with the consequent contact between egg surface and plant cells (Rodriguez-Saona et al. 2002; Frati et al. 2008), or to the high feeding activity of females.

There were no qualitative differences in volatile emissions among the five treatments tested. This has also been shown in

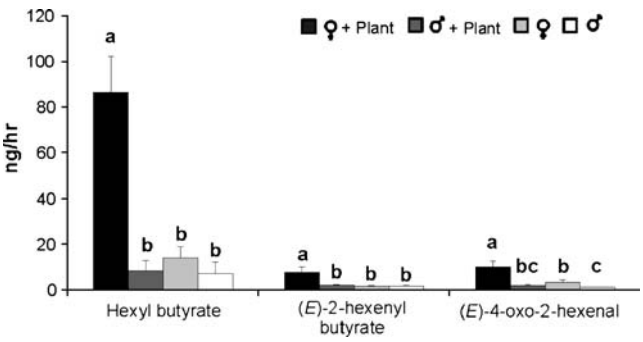

Fig. 2 Release rates of the main volatile compounds collected from mated *Lygus rugulipennis* females (N=8) and males (N=7), with and without host plant (*Vicia faba*). *Each bar* represents a mean ± SE. *Columns with the same letter* indicate means that are not significantly different (P>0.05, ANOVA)

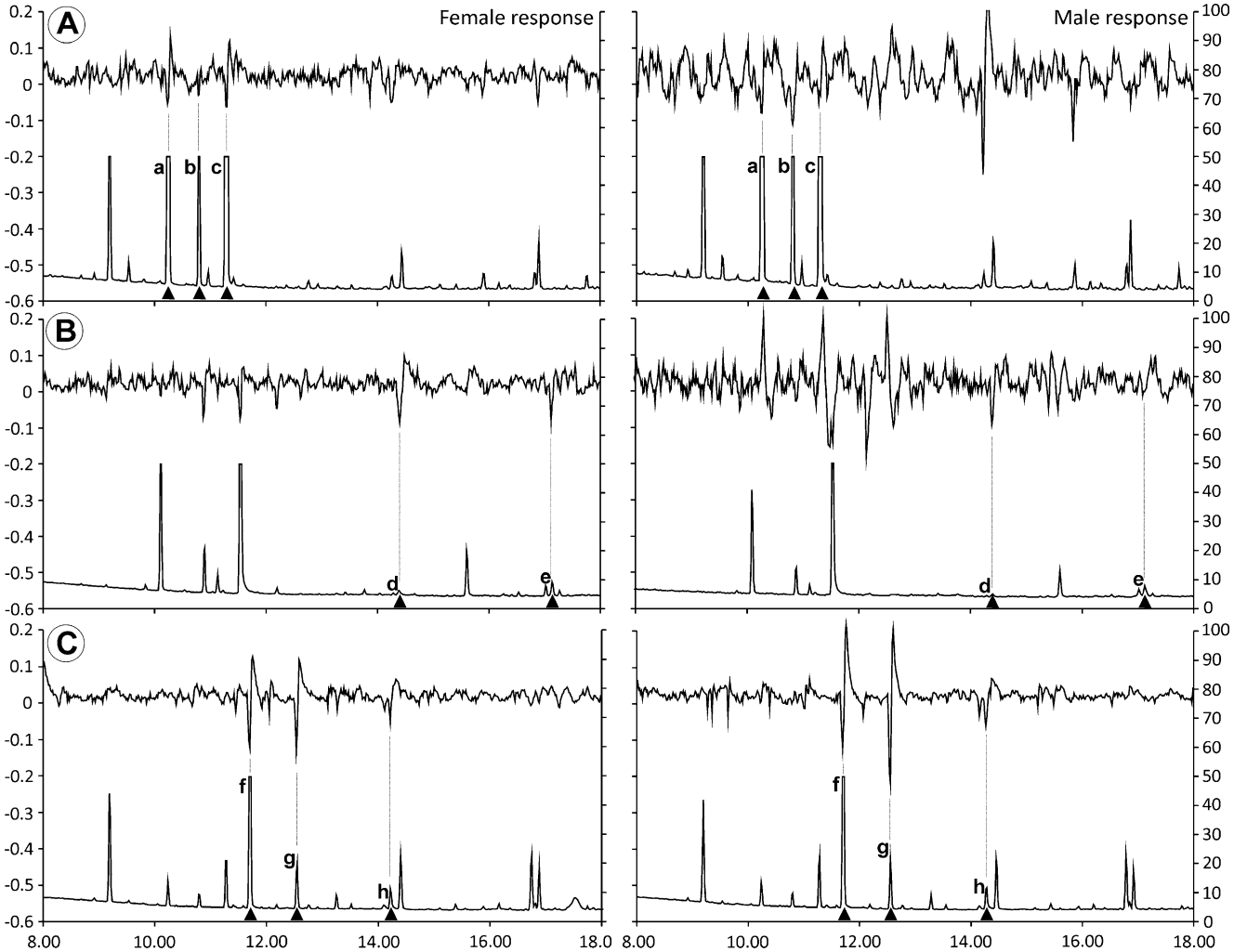

Fig. 3 Coupled gas chromatogram–electroantennograms of *Lygus rugulipennis* females and males to volatile collections from **A** healthy *Vicia faba* plants, **B** plants damaged by females that had been removed, and **C** plants damaged by females still present. *Upper trace*: response (mV, left-side axis title) of antenna; *lower trace*: flame ionization detector response (relative, right-side axis title). Compounds (only those showing electroantennogram activity on three or more preparations are given): *a* (*Z*)-3-hexenyl acetate, *b* 1-hexanol, *c* (*Z*)-3-hexenol, *d* (*E*)-β-caryophyllene, *e* methyl salicylate, *f* hexyl butyrate, *g* (*E*)-2-hexenyl butyrate, *h* (*E*)-4-oxo-2-hexenal

feeding/oviposition damage by *L. hesperus* on maize, cotton, and alfalfa (Rodriguez-Saona et al. 2002; Blackmer et al. 2004), and in other sucking insects feeding on leguminous plants, e.g., the aphid *A. pisum* (Harris) (Homoptera: Aphididae; Du et al. 1998; Guerrieri et al. 2002), greenhouse whitefly *T. vaporariorum* (Homoptera: Aleyrodidae; Birkett et al. 2003), and *N. viridula* (Heteroptera: Pentatomidae; Colazza et al. 2004b). We did, however, observe quantitative changes in emission of hexyl acetate, methyl salicylate, (*Z*)-β-ocimene, (*E*)-β-ocimene, and (*E*)-β-caryophyllene, according to treatment.

Work on maize and cotton plants found that larger amounts of (*Z*)-3-hexenal and (*Z*)-3-hexenyl acetate were emitted by plants infested with the American species *L. hesperus* than by insect-free plants (Rodriguez-Saona et al. 2002). We observed no such difference in emission of these compounds among any of our five treatments. This contrast could be due to the respective infestation levels used; our experiments on *V. faba* infested with the European species *L. rugulipennis* used six adults per plant, whereas Rodriguez-Saona et al. (2002) used the American species *L. hesperus* at an infestation of 30–40 adults per plant. Alternatively, perhaps the slight mechanical damage caused by *L. rugulipennis* feeding or ovipositing may not activate the lipoxygenase pathway, consistent with the data reported for *N. viridula* on *V. faba* (Colazza et al. 2004b).

Compared to control plants, methyl salicylate was released in greater amounts by plants with females or males present, and also by plants on which females had been present. This confirms that the shikimic acid pathway, and in particular methyl salicylate, is induced by feeding by *L. rugulipennis*. However, plants with females present

released greater amounts than plants on which males were present. This suggests that females either feed more actively than males, or that ovipositional damage (in addition to feeding damage) also induces this pathway.

Broad bean treatments also showed quantitative differences in the emission of the terpenoids, (*E*)-β-caryophyllene, (*Z*)-β-ocimene, and (*E*)-β-ocimene, particularly between plants with females present and control plants. An increase in (*E*)-β-caryophyllene emission by damaged plants has been shown to be caused by feeding damage of *Spodoptera littoralis* caterpillars on maize (Turlings et al. 1998), by oviposition of *Xanthogaleruca luteola* on *Ulmus minor* (Wegener et al. 2001), and by feeding and oviposition by *L. hesperus* on cotton and alfalfa (Rodriguez-Saona et al. 2002; Blackmer et al. 2004), and by *N. viridula* on broad beans (Colazza et al. 2004b). In addition, an increase in (*Z*)-β-ocimene and (*E*)-β-ocimene emission has been shown to result from feeding damage in several different systems (Kessler and Baldwin 2001; Blackmer et al. 2004).

Our data demonstrate that the amount of pheromone released by mated *L. rugulipennis* females is enhanced by the presence of broad bean plants, showing that host plants influence the production and/or release of sex pheromone by *L. rugulipennis*, as in other systems (McNeil and Delisle 1989; Landolt et al. 1994; Deng et al. 2004; Yang et al. 2003). Precisely how the host plant influences females remains to be determined.

In GC–EAG analyses of volatiles from healthy plants, EAG responses were recorded from both sexes to (*Z*)-3-hexenol, 1-hexanol, and (*Z*)-3-hexenyl acetate. These green-leaf volatiles could be used by females and males to locate hosts, as suggested by the responses of both sexes to healthy broad beans in a wind tunnel (Frati et al. 2008). In the case of damaged plants without insects, methyl salicylate and (*E*)-β-caryophyllene triggered EAG responses in both sexes. These compounds commonly are released from certain plants during herbivore damage (Chamberlain et al. 2001). Apparently, the greater release of these chemicals by damaged plants does not have an inhibitory effect on *L. rugulipennis*, since both males and females move toward damaged *V. faba* plants in a wind tunnel (Frati et al. 2008). Whether or not the increased release of these chemicals by damaged plants conveys any information to *L. rugulipennis* males and females is unknown.

In the GC–EAG analysis of volatiles released by plants with mated females still on the plant, EAG responses were recorded from both sexes to the three pheromone components, hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal. This suggests that these compounds may have an aggregation function rather than a strict sexual function. Female–female attraction was first proposed in *L. rugulipennis* by Glinwood et al. (2003), and subsequently was confirmed in wind tunnel and vertical open olfactometer experiments, in which the plant-conspecific complex of mated females, attracted females (Frati et al. 2008). An aggregation-type function of the pheromone produced by mated females when on the plant could be beneficial for this insect, since it could help conspecifics find a suitable food source, thus increasing the efficiency of resource use, as well as attracting mates (Dicke and van Loon 2000; Frati et al. 2008).

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Ring-Fluorinated Analog of Methyl Eugenol: Attractiveness to and Metabolism in the Oriental Fruit Fly, *Bactrocera Dorsalis* (Hendel)

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Abstract Oriental fruit fly, *Bactrocera dorsalis* (Hendel), males are highly attracted to the natural phenylpropanoid methyl eugenol (ME). They compulsively feed on ME and metabolize it to ring and side-chain hydroxylated compounds that have both pheromonal and allomonal functions. Side-chain metabolic activation of ME leading to (*E*)-coniferyl alcohol has long been recognized as a primary reason for hepatocarcinogenicity of this compound in rodents. Earlier, we demonstrated that introduction of a fluorine atom at the terminal carbon of the ME side chain significantly depressed metabolism and specifically reduced formation of coniferyl alcohol but had little effect on field attractiveness to *B. dorsalis*. In the current paper, we demonstrate that fluorination of ME at the 4 position of the aromatic ring blocks metabolic ring-hydroxylation but overall enhances side-chain metabolism by increasing production of fluorinated (*E*)-coniferyl alcohol. In laboratory experiments, oriental fruit fly males were attracted to and readily consumed 1,2-dimethoxy-4-fluoro-5-(2-propenyl)benzene (I) at rates similar to ME but metabolized it faster. Flies that consumed the fluorine analog were as healthy post feeding as ones fed on methyl eugenol. In field trials, the fluorine analog I was ~50% less attractive to male *B. dorsalis* than ME.

Keywords Oriental fruit fly · *Bactrocera dorsalis* (Hendel) · Methyl eugenol · 1,2-dimethoxy-4-fluoro-5-(2-propenyl) benzene · Metabolism · Field attraction

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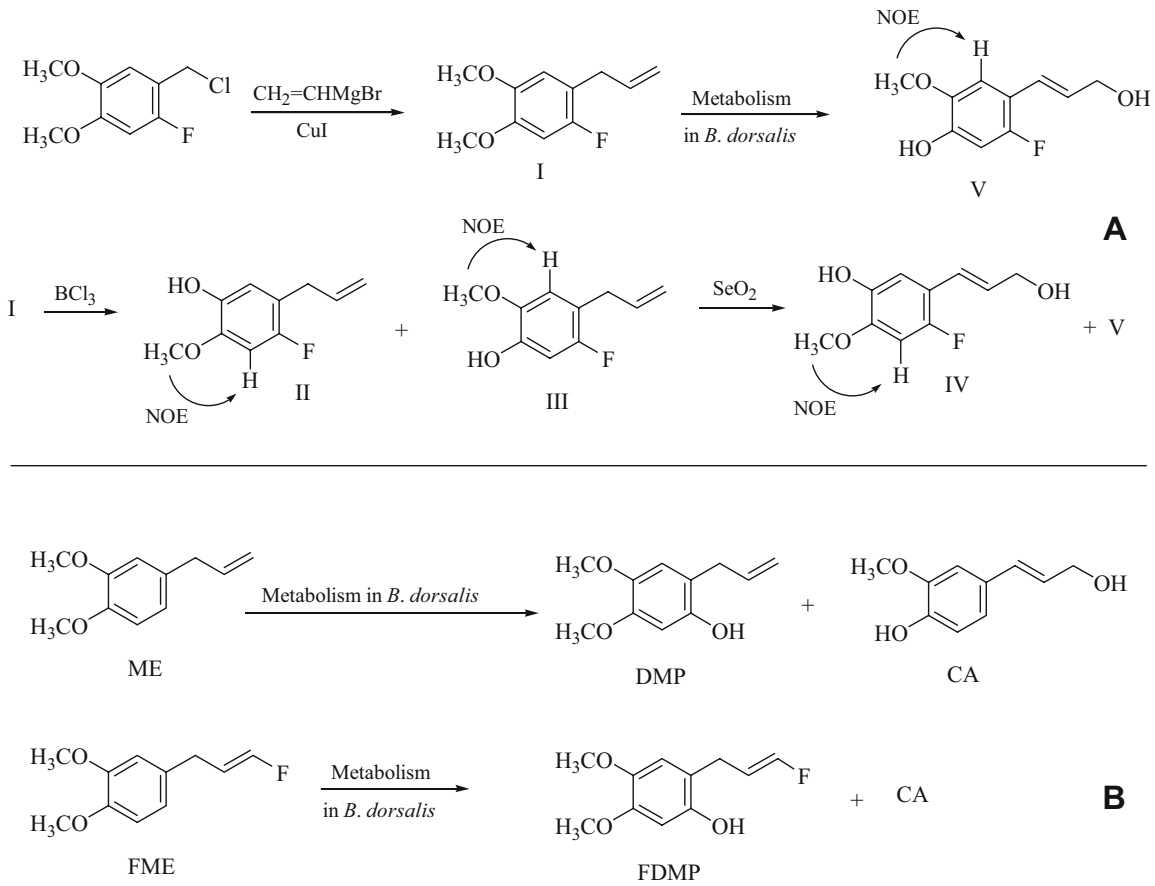
Introduction

The oriental fruit fly, *Bactrocera dorsalis* (Hendel), is a widespread pest of a broad range of tropical, subtropical, and temperate host plants (White and Elson-Harris 1992; US Department of Agriculture 1983). Males of *B. dorsalis* are strongly attracted to and compulsively feed on methyl eugenol (ME, Scheme 1; Steiner 1952), a commonly occurring plant phenylpropanoid (Metcalf 1990). The use of ME in traps and killing stations is an integral part of successful detection, control, and eradication strategies for the oriental fruit fly populations worldwide (Steiner et al. 1965; Koyama et al. 1984). The attraction to and consumption of ME by *B. dorsalis* and sibling species of the *dorsalis* complex, *B. papayae* and *B. carambolae*, have ecological significance in the fly's communication system. Mature



Scheme 1 **A** Synthesis and metabolism of the ring-fluorinated analog I in *B. dorsalis*, and chemical transformation of I to metabolite V. **B** Metabolism of methyl eugenol (*ME*) and the side-chain fluorinated analog of ME (*FME*) in *B. dorsalis*.

males, after feeding on ME, metabolize it primarily to 2-(2-propenyl)-4,5-dimethoxyphenol (DMP) and (*E*)-coniferyl alcohol (CA), which display pheromonal and allomonal properties (Nishida et al. 1988; Tan and Nishida 1996).

A potential problem to the continued use of ME in area-wide pest management programs is the decision by the National Toxicology Program, US Department of Health and Human Services to classify this compound as a carcinogen (National Toxicology Program 1998, 2002). Metabolic activation of ME and production of 1′-hydroxy metabolites through enzymatic side-chain hydroxylation are thought to be primarily responsible for hepatotoxicity and carcinogenicity of ME (Smith et al. 2002). Since early warnings about the carcinogenicity of ME (Miller et al. 1983; Schiestl et al. 1989; Sekizawa and Shibamoto 1982), a number of compounds have been evaluated as attractants for *B. dorsalis* (Metcalf et al. 1975; Mitchell et al. 1985; Khrimian et al. 1993, 1994; Liquido et al. 1998). One promising replacement for ME is a fluorine analog, (*E*)-1,2-dimethoxy-4-(3-fluoro-2-propenyl)benzene (FME, Scheme 1), which in limited field tests was as attractive to *B. dorsalis* as methyl eugenol (Khrimian et al. 1994). It is also more persistent than ME and, remarkably, about twice as active as the corresponding *Z* isomer (Khrimian et al. 1994; Liquido et al. 1998). This compound also displays a reduced propensity to oxidative biotransformations, specifically a side-chain hydroxylation in *B. dorsalis* (Khrimian et al. 2006), which is linked to the hepatotoxicity and carcinogenicity of ME (Smith et al. 2002). While the mechanism is still unclear, FME showed reduced toxicity and reduced recombinagenicity in yeast deletion assays compared to ME (Brennan et al. 1996). In the current paper, we present the results of our studies on behavioral bioassays and metabolism of yet another fluorinated ME analog, 1,2-dimethoxy-4-fluoro-5-(2-propenyl)benzene (I, Scheme 1). Of particular interest is how the fluorine introduction at a key metabolic hydroxylation site of ME would affect the overall biotransformation in oriental fruit fly. In addition, we were interested to quantify the attractiveness of compound I relative to its analogs ME and DMP, which also was highly attractive to *B. dorsalis* males (Nishida et al. 1988). There is ample information in the literature demonstrating that replacement of a hydroxy group with fluorine in the biologically active compounds may be successful (Welch 1987, 1991). In

addition, we expected such fluorine substitution to render an improved volatility compared to DMP, which may be beneficial for a fruit fly attractant.

Methods and Materials

Analytical Procedures ^{1}H NMR spectrum of I was recorded at 300 MHz in $CDCl_3$ with TMS as an internal standard on a Bruker QE-300 spectrometer. ^{1}H NMR spectra of other compounds were obtained at 500 MHz on a Bruker DRX-500 using TMS as an internal standard. Chemical shifts are reported in δ units, and coupling constants in Hz. GC analyses were performed on a Shimadzu 17A (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) gas chromatograph equipped with a flame ionization detector, an auto sampler AOC-20s and auto injector AOC-20i, and an HP-5 capillary column (30 m×0.25 mm×0.25 μm film). Hydrogen was used as carrier gas at 1 ml/min. Column temperature was maintained at 100°C for 2 min and then raised to 260°C at 10°C/min. Electron-ionization (EI) mass spectra were obtained at 70 eV with an Agilent Technologies 5973 mass selective detector interfaced with a 6890 N GC system equipped with a 30 m×0.25 mm i.d.×0.25 μm film HP-5MS column. Column temperature was maintained at 80°C for 1 min and then raised to 270°C at 10°C/min. Helium was used as carrier gas at 1 ml/min. TLC analyses were conducted on Whatman AL SIL G/UV plates using hexanes/ethyl acetate as a mobile phase with a 20% ethanol solution of phosphomolybdic acid and/or UV used for visualization of spots. Flash chromatography was carried out with 230–400 mesh silica gel (Fisher Scientific, Fair Lawn, NJ, USA).

Chemicals All reagents and solvents were purchased from Aldrich Chemical Co. unless otherwise specified.[1] Methylene chloride and hexamethylphosphoramide (HMPA; caution, it is a carcinogen!) were distilled from calcium hydride, and tetrahydrofuran (THF) was dried by distillation from sodium benzophenone-ketyl. Methyl eugenol metabolites, CA and DMP, were prepared from ferulic acid (Quideau and Ralph 1992) and 3,4-dimethoxyphenol (Benbow and Katoch-Rouse 2001), respectively. FME was prepared as described (Khrimian et al. 2006). 1-Chloromethyl-2-fluoro-4,5-dimethoxybenzene was prepared following a procedure of Furlano and Kirk (1986). The crude chloride was used in a copper-catalyzed Grignard coupling without further purification as described (Khrimian et al. 1993).

[1] Mention of commercial products does not constitute an endorsement by USDA.

1-Fluoro-4,5-dimethoxy-2-(2-propenyl)benzene (I) A solution of 1-chloromethyl-2-fluoro-4,5-dimethoxybenzene (1.62 g, 7.92 mmol) in dry THF (8 ml) was introduced under N_2 to a three-neck flask and cooled to −10°C. A solution of copper(I) iodide (150 mg, 0.79 mmol) in dry HMPA (6 ml) was added at once, followed by vinylmagnesium bromide (11.88 mmol; 11.88 ml of 1.0 M in THF) delivered slowly at −5–(−10)°C. The mixture was stirred at that temperature range for 30 min, then slowly warmed to 25°C over ~4 hr. The mixture was poured into a saturated ammonium chloride solution and extracted with ether (3×50 ml). The combined organic extracts were washed three times with saturated NH_4Cl and dried with Na_2SO_4. After evaporation of the solvent, the remainder was distilled in vacuum at 62–64°C/0.3 mm Hg to give I (800 mg, 52%) of 96% purity. Flash chromatography of this material on SiO_2 with hexanes/ethyl acetate, 8:1, gave I (600 mg) of 99% purity. ^{1}H NMR: 3.34 (dd, 2H, H-1′, J= 6.3, 1.2), 3.84 (s, 6H, OCH_3), 5.07 (m, 2H, H-3′), 5.94 (m, 1H, H-2′), 6.62 (d, $^3J_{HF}$=10.8, H-3), 6.65 (d, $^4J_{HF}$=7.5, H-6). GC-MS (EI): 196 (M^+, 100%), 181 (85), 169 (49), 165 (75), 125 (75), 109 (67).

4-Fluoro-2-methoxy-5-(2-propenyl)phenol (II) and 5-fluoro-2-methoxy-4-(2-propenyl)phenol (III) Fluorinated methyl eugenol I (980 mg, 5 mmol) was stirred with boron trichloride (6 ml of 1 M in CH_2Cl_2, 6 mmol) in dry CH_2Cl_2 (10 ml) at 0 to 25°C under N_2. After 24 hr, or when TLC showed no starting material present, the mixture was poured into ice-water and extracted with CH_2Cl_2. The combined extracts were washed with a saturated solution of sodium bicarbonate to pH ~7, then dried with sodium sulfate and concentrated. Flash chromatography with hexanes/ethyl acetate, 4:1 to 1:1, afforded a mixture of phenols II and III in a 57:43 ratio (592 mg, 65%). Phenols II and III were not separated by TLC but showed a baseline separation by GC on a DB-5 column, with phenol III being the first isomer to elute. The assignment of the isomeric phenol was done by ^{1}H NMR. GC-MS (*m*/*z*, %): II—182 (100, M^+), 167 (21), 155 (20), 151 (11), 149 (21), 139 (10), 121 (10), 109 (15), 91 (14). III—182 (100, M^+), 167 (32), 155 (18), 149 (15), 139 (8), 121 (11), 109 (15), 91 (11). ^{1}H NMR (500 MHz, $CDCl_3$): 3.28 (d, J=6.5, CH_2, II), 3.31 (d, J=6.5, CH_2, III), 3.82 (s, OCH_3, II), 3.83 (s, OCH_3, III), 5.04 (m, CH=), 5.36 and 5.67 (OH), 5.91 (m, CH_2=), 6.57 (d, $^3J_{HF}$=10.6, H-3, II), 6.61 (d, $^4J_{HF}$=6.9, H-3, III), 6.64 (d, $^3J_{HF}$=10.6, H-6, III), 6.71 (d, $^4J_{HF}$=6,9, H-6, II).

(E)-4-Fluoro-5-(3-hydroxy-1-propenyl)-2-methoxyphenol (IV) and (E)-5-fluoro-4-(3-hydroxy-1-propenyl)-2-methoxyphenol (V) Selenium dioxide (200 mg, 1.8 mmol) was dissolved in hot water (0.2 ml; ~80°C) and treated with a solution of phenols (II+III, 318 mg, 1.75 mmol) in dioxane (1.6 ml). The resulting mixture was heated in a water bath

(~85°C) for 15 min, then diluted with water (8 ml) and extracted with CH_2Cl_2 (4×5 ml). The combined organic extracts were washed with water (2×2 ml), brine (2×2 ml) and then dried with $MgSO_4$. The solution was concentrated, and the remainder was flash-chromatographed with hexanes/ethyl acetate, 3:4, to furnish some unreacted starting material (53 mg), then phenol IV (20 mg, R_f 0.44, hexanes/ethyl acetate, 1:2), a mixture of IV and V (16 mg) and, finally, phenol V (11 mg, R_f 0.38, hexanes/ethyl acetate, 1:2,). GC-MS (*m*/*z*, %): IV (Rt 14.27 min) – 198 (77, M^+), 155 (100), 142 (36), 140 (9), 137 (14), 127 (8), 121 (9), 109 (18); V (Rt 13.83 min) – 198 (71, M^+), 155 (100), 142 (36), 140 (8), 137 (12), 127 (10), 109 (16).^{1}H NMR (500 MHz, $CDCl_3$ + CD_3OD, IV): 3.77 (s, OCH_3), 4.17 (dd, *J*=6.0, 1.0, CH_2), 6.15 (dt, *J*=16.0, 5.5, H-2′), 6.49 (d, $^3J_{HF}$=11.5, H-3), 6.57 (br. d, *J*=16.0, H-1'), 6.87 (d, $^4J_{HF}$= 7.0, H-6). ^{1}H NMR (500 MHz, CD_3CN, V): 3.84 (s, OCH_3), 4.03 (dd, *J*=6.0, 1.5, CH_2), 6.25 (dt, *J*=16.0, 6.0, H-2′), 6.59 (d, $^3J_{HF}$=11.5, H-6), 6.63 (br. d, *J*=16.0, H-1'), 7.04 (d, $^4J_{HF}$=7.5, H-3).

Insects Laboratory reared oriental fruit flies (strain: Punador initiated in 1988–1989 and monitored by mating and survival tests) were obtained from the USDA-ARS-PBARC rearing facility in Honolulu, Hawaii. Pupae (60 ml) were placed in a screened aluminum cage (30×30×30 cm) and allowed to emerge. Flies were given water, sugar, and hydrolyzed yeast protein and held at 25–26°C, 50–70% relative humidity and a 12/12 hr L/D cycle before using in laboratory trials. Field bioassays were conducted with sterile laboratory reared oriental fruit fly, obtained as pupae, from a laboratory colony at the USDA-ARS-PBARC rearing facility in Honolulu, HI. Emerged adults were kept in an insectary at 24–27°C, 65–70% RH, and a photoperiod of 12:12 hr (L:D). Adults were fed water and a diet of three parts sucrose, one part protein yeast hydrolysate (Enzymatic, United States Biochemical Corporation, Cleveland, OH), and 0.5 part torula yeast (Lake States Division, Rhinelander Paper Co., Rhinelander, WI). Ten–eleven-day old, sexually mature, flies were used in the field studies.

Bioassays

Laboratory Feeding and Metabolism In Lab Trial 1, 50 laboratory-reared mature (9–10-day-old) oriental fruit fly males were placed in five ~1-l plastic containers (10/container) equipped with water inserts and screened covers. Flies were offered 10 μl of ME (2×5 μl) on a slip of filter paper (25×5 mm) taped to a blank water vial. Another 50 flies were offered the fluorine analog I (10 μl/cage), and the last group of 50 males were presented with a filter paper containing 10 μl water. Behavioral observations (landing and feeding on the source) were recorded every 5 min for 1 hr, then filter papers were removed, water containers were introduced, and the flies were left overnight with a sugar cube on the cover available for consumption. Numbers of dead flies was counted at 24 and 48 hr. Eighteen hours post feeding, the rectal glands of ten flies each fed with ME and I (cages where the landings occurred most), as well as ten control flies were dissected and placed in conical plastic vials with 20 μl ethanol. The glands were crushed with a wire rod, sealed with parafilm and centrifuged at ~4,000 rpm for 10 min. The supernatants were diluted in the same vials with ethanol to 100 μl and analyzed by GC-MS in splitless mode by injection of 1 μl solutions. External standards of CA, DMP, and V were used for quantification. The remainders of the dissected flies were individually placed in 20 μl ethanol, crashed, then flash-chromatographed on a short SiO_2 column (30×5 mm) with hexanes/ethylacetate, 3:1. The fractions containing unmetabolized ME and fluorine analog I were further combined, concentrated to 1 ml, and quantified by GC using external standards.

Lab Trial 2 was conducted analogously to Lab Trial 1 with the exception of the treatments presented which were 1 μl of ME (10 μl of 10% *v*/*v* hexane solution), 1 μl of fluorine analog I, and water as control. In Lab Trial 3, the doses of treatments were reduced to 0.1 μl. No dissections and metabolism studies were conducted in Lab Trials 2, and 3.

Field Attractiveness and Release Rate

Dispenser Preparation and Trapping For each test, cotton dental wicks (9 mm diam × 14 mm long) were treated with aliquots of a hexane-acetone stock solution (2:1 *v*/*v*) containing a test compound. Three chemicals, fluorine analogs I, ME, and FME, were tested in Field Trial 1 while four chemicals, I, ME, FME, and DMP were tested in Field Trial 2. Each wick was treated with 250 μl of the stock solution, a volume calculated to deliver ca. 10 mg of a test chemical. After allowing the solvent to evaporate (20–25 min), wicks were enclosed individually in screen-covered plastic baskets (to prevent feeding consumption by oriental fruit fly males). By using metal clips, baskets that contained wicks were hung inside Jackson traps. Each Jackson trap had a sticky basal insert and two 2.5-cm dog collar strips (Bansect® flea and tick collar for dogs, ®ConAgra Pet Products, Richmond, VA, USA) containing a knock down toxicant (15% Naled by weight; ~0.38 g/strip). Bioassays with releases of 10–11-day old laboratory-reared oriental fruit fly adults were conducted in a macadamia (*Macadamia integrifolia* Maiden and Betche) orchard in the vicinity of Hilo, Hawaii. A low-density population of wild oriental fruit flies subsisting on wild hosts inside and outside this orchard occurred naturally. The experimental design was randomized complete block with six (Field Trial 1) or five

(Field Trial 2) replications. The interblock distance was 10 m, with treatments in each block placed 8 m apart. Approximately 6,000 sterile oriental fruit fly males were uniformly released per block at the start (5 Sept., 1995 for Field Trial 1; 1 Nov., 1995 for Field Trial 2) of each of two successive 24-hr periods. After 24 hr, inserts with trapped adults from each trap were recovered, and new inserts were placed in each trap. All traps were gathered and moved to a new trapping grid of exactly the same configuration at least 500 m away (moving the traps to another trapping grid was done to approximate similar, uniform density of adults in the trapping grid for 0–24 and 24–48 hr). After 48 hr, wicks and inserts were recovered from each trap and immediately brought back to the laboratory. The total number of trapped oriental fruit fly males was determined for each retrieved insert.

Weathering of Wicks and Residue Analyses At the time of the initial wick treatment for Field Trial 2, five treated wicks of each treatment (time 0 wicks) were placed in individual glass vials, sealed with Teflon tape, and frozen. Concurrent with the distribution of treatments in Field Trial 2 (described above), the same number of additional traps of each treatment were emplaced in a randomized complete block wick weathering grid. Wicks recovered from these grids after 24 hr, together with time 0 wicks and wicks collected after 48 hr from the trap catch grid, were used to estimate the amount of compound that had volatilized from the wicks during 0–24 hr and 24–48 hr of Field Trial 2. As with wicks used in the bioassays, weathering wicks were enclosed in screen-covered baskets that were hung inside Jackson traps. Following retrieval at 24 and 48 hr, wicks were placed individually in 25-ml glass vials, sealed with Teflon tape, and shipped to Beltsville, MD, USA, for residual analyses. Gas chromatography-quality acetone was added to each vial (15 ml), vials were stoppered, and wicks were allowed to steep overnight at 25°C. A predetermined amount of an internal standard, 1,2-dimethoxy-4-ethylbenzene (synthesized following Khrimian et al. 1993) was added to each vial, and the extracts were analyzed by gas chromatography.

Data Analysis Mean data from the metabolism study (Lab Trial 1) were analyzed using PROC GLM followed by a Tukey's HSD test for mean separation. Significant differences were determined at the $P<0.05$ level. Analysis was run on SAS version 8.2 (SAS Institute 1990). Mean data from the feeding study were analyzed with a two-way ANOVA. These analyses were conducted by using STATISTICA (Statsoft 2001). Trap catch results from the field bioassays were square root transformed ($\sqrt{[x+0.5]}$; Sokal and Rohlf 1981) and then subjected to ANOVA with Tukey's HSD used for means separation (SAS Institute 2002). Treatment volatilization was analyzed by ANOVA for the 0–24-hr and 24–48-hr periods, with Tukey's HSD used for means separation (SAS Institute 2002).

Results

Syntheses of Ring-fluorinated Analog I and Metabolite V We straightforwardly synthesized the targeted fluorinated benzene I (Scheme 1, panel A) from 1-chloromethyl-2-fluoro-4,5-dimethoxybenzene by using a coupling proce-

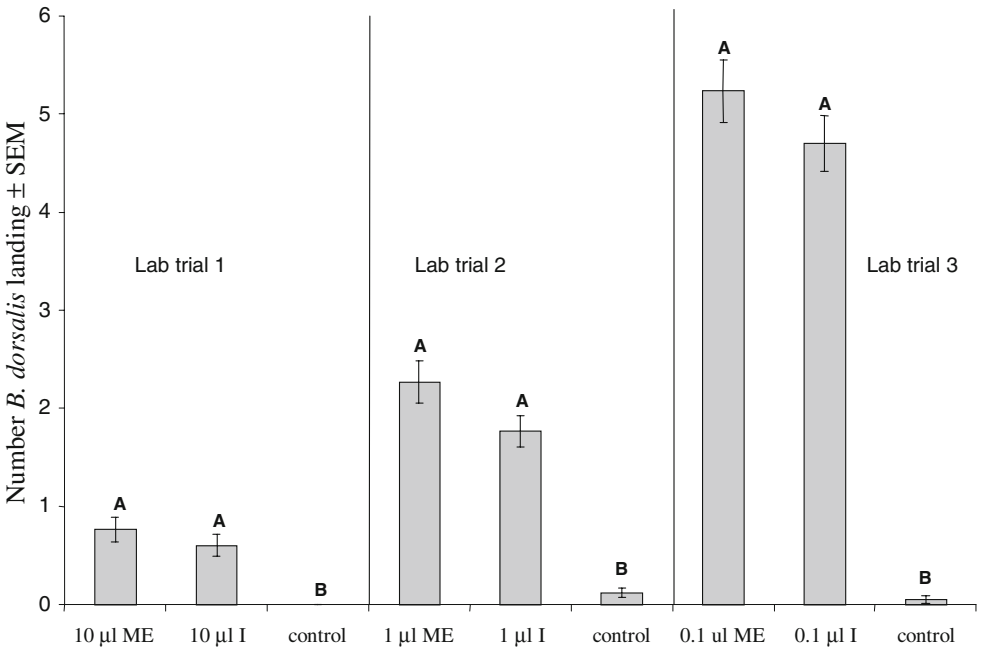


Fig. 1 Landing and feeding of *B. dorsalis* males in a cage experiments with ME and fluorine analog I. Ten males were offered two concentrations of both attractants in five replicates. A two-way ANOVA showed a significant effect of dose ($df=2.24$; $F=76.6$, $p<0.001$) but no differences between treatments within trials, ME and I ($df=1.24$; $F=1.91$; $p=0.18$). Treatment dose was nonsignificant ($df=2.24$; $F=0.16$, $p=0.85$). *Means followed by the same letter* are not significantly different. (Statsoft Inc, 2001)

dure developed earlier for alkyl-substituted analogs of methyl eugenol (Khrimian et al. 1993). Fluorinated benzene I was converted to V with a two-step procedure depicted in Scheme 1, panel A. First, compound I was demethylated with boron trichloride in methylene chloride (Dean et al. 1966) to give a 57:43 mixture of two phenols, baseline separated by GC on a DB-5 column but not by TLC. Thus, the prospect of isolating the individual phenols was uncertain, and their structural assignment presented a certain challenge. Fortuitously, signals from the aromatic protons of the two isomeric phenols were clearly separated in the ^{1}H NMR spectrum of the mixture recorded at 500 MHz, which allowed us to apply a nuclear Overhauser effect (NOE) for their assignments. A NOE difference spectrum obtained by irradiating CH_3O resonance (at about the same frequency for both isomeric phenols) showed an enhancement of two signals at δ 6.57 (d, J=10.6 Hz) and 6.61 (d, J=6.9 Hz). Based on the literature evidence of larger proton-fluorine coupling constant in the *ortho* position ($^3J_{HF}$) compared to that of in the *meta* position ($^4J_{HF}$), and the actual values of these interactions (Silverstein et al. 1980), we assigned the first doublet (of higher intensity) to the proton on C-3 of phenol II and the second to the proton on C-3 of phenol III. Conversely, the signals at δ 6.64 (d, J=10.6 Hz) and 6.71 (d, J=6.9 Hz) were assigned to protons on C-6 of phenols III and II, respectively. Thus, phenol III, needed for the completion of the synthesis of the metabolite V, was a minor component in the mixture. In the last step, phenols II and III were oxidized with selenium dioxide in an aqueous dioxane (Pan et al. 1980) to give a mixture of diols, from which pure IV and V were isolated by flash chromatography. The NOE difference technique described above was used again for structural assignments. In the case of V, irradiation of CH_3O led to the enhancement of the doublet at δ 7.04 ($^4J_{HF}$=7.5 Hz) from the proton on C-3, positioned *meta* to the fluorine atom, whereas in diol IV a NOE effect from CH_3O resonance occurred on the proton in the *ortho* position to fluorine (δ 6.49 d, $^3J_{HF}$=11.5 Hz). *Trans*-configuration of the double bonds in both IV and V was evident from $^3J_{HH}$=16.0 Hz and concurrent with the chemistry of SeO_2 oxidation of 3-phenylpropenes (Pan et al. 1980). Synthetic diol V, based on a GC retention time and mass-spectrum, matched the compound found in rectal glands of oriental fruit fly males fed with I. It was also used as a standard for quantitative analysis of gland extracts.

Feeding and Metabolism In all three experiments (Fig. 1) conducted with different doses, there was no statistically significant difference between fluorine analog I and ME in

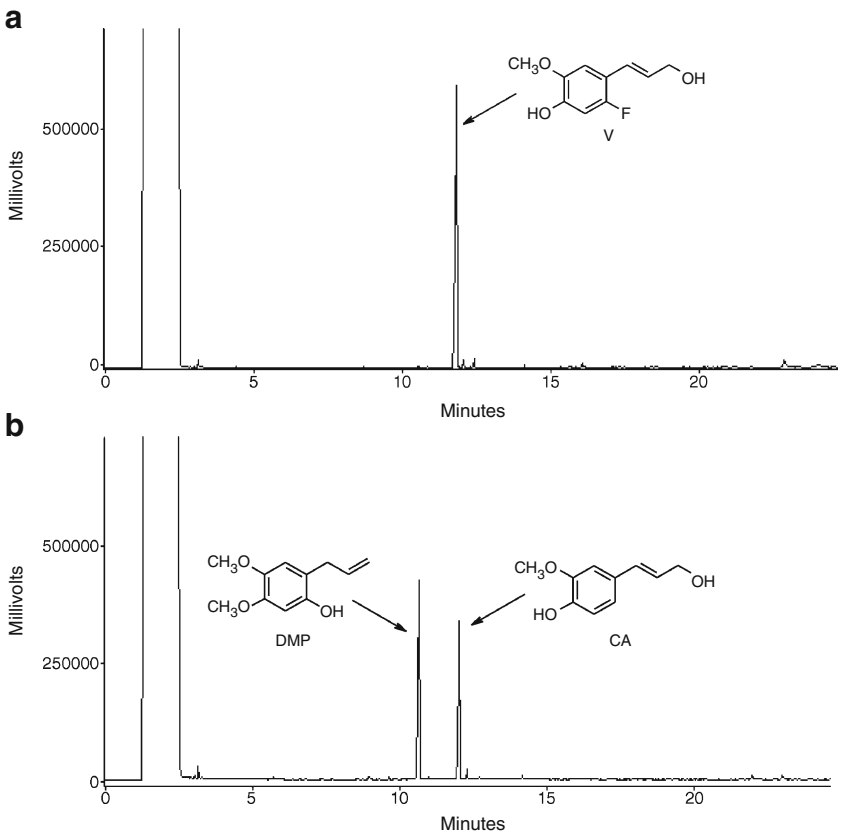


Fig. 2 Gas chromatograms (*HP-5, FID*) of gland extracts of individual oriental fruit fly males fed with: **A** fluorine analog I (Table 1, species # 3), and **B** methyl eugenol (Table 1, species # 1)

Table 1 Amounts of metabolites and unmetabolized ring-fluorinated analog I and ME in oriental fruit fly males from Lab Trial 1

Fluorine analog I				ME				
Insect no.	Metabolite V, μg[a]	Unmetabolized I, μg[b]	V, molar %	Metabolites, μg		Unmetabolized ME, μg[b]	CA, molar %	Total metabolites, molar %
				CA[a]	DMP[a]			
1	64.9	141.5	45.4	16.1	17.3	55.3	18.3	36.5
2	28.2	26.4	51.4	16.3	19.6	302.7	4.8	10.1
3	121.2	135.0	47.1	21.8	14.8	2.1	57.9	94.4
4	52.4	75.1	40.8	24.0	24.6	285	7.2	14.0
5	74.2	26.6	73.4	43.1	20.3	276.5	12.6	18.1
6	99.6	48.4	67.1	4.2	4.2	162.9	2.4	4.7
7	26.6	1.5	94.6	10.6	9.0	66.6	12.3	22.0
8	31.9	1.5	95.4	7.3	4.8	53.7	11.1	17.8
9	72.5	34.0	67.9	16.0	14.6	112.6	11.2	20.6
10	78.3	68.3	53.2	16.8	8.2	54.2	21.2	30.8
Total	650	558		176	137	1372		
Mean ± SEM	65.0±9.9	55.8±15.7	63.6±6.2a[c]	17.6±3.4	13.7±2.2	137.2±35.5	15.9±5.0b[c]	26.9±8.1b[c]

[a] GC analysis of glands
[b] GC analysis of other body parts
[c] Means followed by different letters are significantly different at the 0.05 level, PROC GLM, Tukeys test (SAS Institute 1990)

male *B. dorsalis* landing and feeding on the source (F=1.91; P=0.18). However, there was a difference between doses (df=2.24; F=76.6, P<0.001), with the most landings recorded in Lab Trial 3 (0.1 μl, ~5 landings in average out of 10). The least number of landings were observed in Lab Trial 1 (10 μl, <1 landing), and Lab Trial 2 (1.0 μl) was in the intermediate position (~2 landings). No fly landings were observed in the control cage in Lab Trial 1, and landings in control cages in Lab Trial 2 and 3 were less than in cages containing I and ME (F=76.6; P<0.001). Flies fed with compound I did not suffer significantly higher levels of mortality than those fed with ME. No mortalities were observed after 24 and 48 hr in low-dose Lab Trials 2 and 3 with either treatment. In Lab Trial 1, two flies fed with compound I were dead after 24 hr and total of five flies were dead after 48 hr. In the same high-dose experiment, the numbers of dead flies fed with ME after 24 and 48 hr were five and eight, respectively.

Results of chemical analyses of rectal glands and remaining body parts of ten flies from Lab Trial 1 are presented (Fig. 2, Table 1). A representative gas-chromatogram of a gland extract of an individual *B. dorsalis* male fed with I (Fig. 2, panel A) reveals essentially a single compound that matches the synthetic diol V by retention time and mass-spectrum. As expected, the flies fed with ME produced the well-described metabolites, coniferyl alcohol and 4,5-dimethoxy-2-(2-propenyl)phenol (Fig. 2, panel B). Unmetabolized test chemicals were not found in rectal glands but were present in the extracts of fly remains after dissection. No phenylpropanoids were found in the control flies. As shown in Table 1, total intake of test materials by ten flies offered 10 μl of each I or ME were similar: 1208 (650 +558) μg for compound I and 1509 (137+1372) μg for ME. However, a remarkable difference between the treatments was observed in the amounts of metabolites relative to intakes. At this feeding level, *B. dorsalis* males were able to metabolize, after 18 hr, on average ~64% of the fluorine analog I and only ~27% of ME (~16% of the ME intake was converted to CA). It appears, however, that at a lower consumption level (20–40 μg) flies can almost completely metabolize both compounds (Table 1, rows 7, 8 for I, and row 3 for ME).

Field Bioassay Trap capture of males from field bioassays are presented in Fig. 3 (Field Trial 1) and Fig. 4 (Field Trial 2). There were differences in trap captures by treatment in Field Trial 1 at both 24 hr (F=23.7, df=2, 15, P<0.001) and at 48 hr (F=22.2, df=2, 15, P<0.001); and in Field Trial 2 at both 24 hr (F=14.5, df=3, 16, P<0.001) and at 48 hr (F=41.5, df=3, 16, P<0.001). In Field Trial 1, at both 24 and 48 hr, there was no significant difference in captures between traps baited with ME and traps baited with FME. However, catches at traps baited with I were statistically less than at traps baited with either ME or FME. In Field Trial 2, at both 24 and 48 hr, there was again no significant difference in catches between traps baited with ME or with FME. Captures at traps baited with I were significantly less than at traps baited with methyl eugenol (but not FME) at 24 hr and were significantly less than at traps baited with either ME or FME at 48 hr. Catches at traps baited with

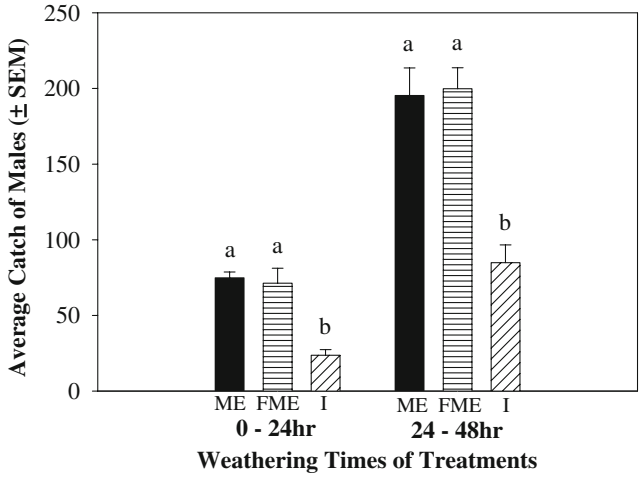


Fig. 3 Field Bioassay 1, September 1995. Average catch of male oriental fruit fly adults 0–24 and 24–48 h after field deployment of Jackson traps baited with methyl eugenol (*ME*), ring-fluorinated analog (*I*), and side-chain fluorinated analog (*FME*). *Columns within the same time period having the same letter* are not significantly different at the α=0.05 level

DMP were significantly less than catches with all other treatments at both 24 and 48 hr. There were differences in volatilization by treatment in both the 0–24 hr (F=289.4, df=3, 16, P<0.001) and 24–48 hr (F=116.8, df=3, 16, P<0.001) time periods. Release rates of I and ME from cotton wicks were not significantly different at either 24 or 48 hr, whereas release of FME was significantly lower than those of I and ME at both 24 and 48 hr. Finally, the release of DMP was significantly lower than those of any other treatments at both time intervals.

Discussion

Our laboratory studies demonstrated that the ring-fluorinated analog I is perceived by *B. dorsalis* males as an analog of natural phenylpropanoid methyl eugenol. In close range experiments, flies were attracted to and fed on analog I and methyl eugenol at similar rates. At the highest concentration of test materials in cage experiments, flies rarely touched the source. This behavior is similar to insect avoidance, observed under field conditions, of traps baited with a large amount of a pheromone (Zhang and Amalin 2005). Although few flies directly contacted the filter papers, they still absorbed appreciable amounts of vaporized I and ME. Consumption of fluorine analog I did not result in an increased mortality in *B. dorsalis* males compared to flies fed with ME, which has been reported to cause death in the oriental fruit fly when ingested in large amounts (Steiner 1952). Thus, the fitness of *B. dorsalis* males evidently is linked (among other factors) to their ability to efficiently metabolize phenylpropanoids.

Introduction of the fluorine atom on the aromatic ring of ME at a position which undergoes enzymatic hydroxylation (Scheme 1, panel B) resulted in a complete blocking of this biotransformation pathway. While this was not particularly surprising, the observation that blocking one pathway did not result in an overall slowing of metabolism was interesting. Oriental fruit fly males demonstrated a remarkable ability to shift metabolism of the consumed fluorine analog I through a side-chain hydroxylation pathway (Smith et al. 2002), leading to the fluorinated (*E*)-coniferyl alcohol V. In fact, the rate of metabolism of I was about twice that of ME under our experimental conditions (Table 1, last columns). Our previous studies with FME (Scheme 1, panel B) showed that the fluorine introduction slowed overall metabolism and specifically impeded formation of CA (Khrimian et al. 2006). Thus, the incorporation of the fluorine atom at the aromatic ring and at the

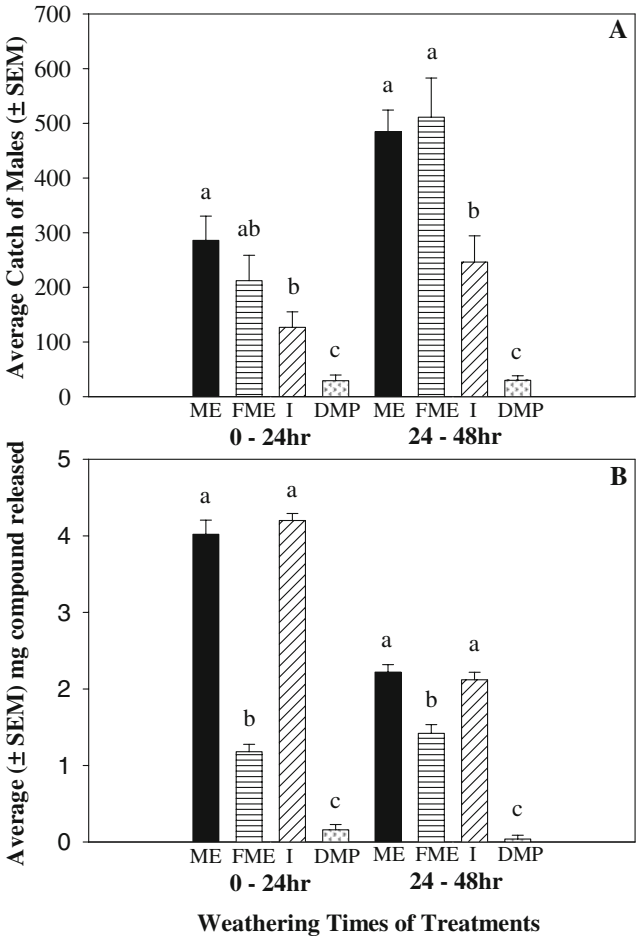


Fig. 4 Field Bioassay 2, November 1995. Average catch of male oriental fruit fly adults (**A**) and release of test chemicals (**B**) 0–24 and 24–48 h after field deployment of Jackson traps baited with methyl eugenol (*ME*), ring-fluorinated analog (*I*), side-chain fluorinated analog (*FME*), and dimethoxyphenol (*DMP*). *Columns within the same time period having the same letter* are not significantly different at the α=0.05 level

terminal carbon atom of the double bond of ME had opposite effects on the side-chain metabolic hydroxylation.

Although *B. dorsalis* males were attracted strongly to and fed on fluorine analog I in a cage experiment, field attractiveness of male oriental fruit fly to I was markedly lower than to ME. In both field bioassays, traps baited with compound I captured less than 50% of flies caught in traps baited with ME. Release rates of fluorine analog I and ME were similar (Fig. 4), so trap catches provide a valid comparison of field attractiveness of these two compounds. In contrast, the evaporation of DMP from a cotton wick was substantially (~30 times) lower compared to I, making a linkage of trap catches to attractiveness tenuous. Even at a much slower release rate, however, the average number of males caught in traps baited with DMP was only 4–8 times lower than that in analog I-baited traps. Thus, a fluorine substitution for the hydroxy group at the aromatic ring of DMP increased the volatility but did not improve attractiveness to *B. dorsalis*. Metcalf et al. (1975, 1981) studied molecular parameters of attractants and olfaction in *B. dorsalis* that involved a number of substituted *ortho*-dimethoxybenzenes. Several postulates were derived from those studies: (a) unshared electron pairs of the *ortho*-dimethoxy groups were essential for receptor depolarization, yet the methoxy group in *para* position to allyl group of ME seemed to be of a primary importance; (b) electron-donating side-chain groups produced stronger attractants than those withdrawing electrons.

We did not find in the literature any activity correlations with the substitution pattern of DMP and fluorine analog I, but because the fluorine atom is isosteric with hydrogen (and being such has been widely pursued for altering the properties of biologically active substances), it seems that it is the electronic factor of fluorine substitution that is responsible for a reduced attractiveness of analog I compared to ME. A strong electronegative effect of the fluorine atom should decrease electron density about the CH_3O groups in analog I but would primarily affect the methoxy group in the *para*-position to F (*meta* to allyl group), thus diminishing but not abolishing the attraction to *B. dorsalis*. In any case, a moderate field attractiveness combined with the propensity to side-chain metabolic oxidation (linked to a carcinogenicity) makes the analog I an unlikely replacement for ME as an attractant for the oriental fruit fly. We, however, were pleased with the performance of FME in two short field bioassays, which confirmed our earlier findings (Khrimian et al. 1994; Liquido et al. 1998) that this compound is not only equally attractive to *B. dorsalis* but also has added value as a more persistent lure.

In summary, we developed a ring-fluorinated analog of methyl eugenol that is highly attractive to the oriental fruit fly males in a laboratory cage experiment but is only moderately active in the field. This new fluorine analog is metabolized faster than methyl eugenol after being consumed by *B. dorsalis* males and produces almost exclusively fluorinated (*E*)-coniferyl alcohol.

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NMR Metabolomics of Thrips (*Frankliniella occidentalis*) Resistance in *Senecio* Hybrids

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Abstract Western flower thrips (*Frankliniella occidentalis*) has become a key insect pest of agricultural and horticultural crops worldwide. Little is known about host plant resistance to thrips. In this study, we investigated thrips resistance in F_2 hybrids of *Senecio jacobaea* and *Senecio aquaticus*. We identified thrips-resistant hybrids applying three different bioassays. Subsequently, we compared the metabolomic profiles of these hybrids applying nuclear magnetic resonance spectroscopy (NMR). The new developments of NMR facilitate a wide range coverage of the metabolome. This makes NMR especially suitable if there is no a priori knowledge of the compounds related to herbivore resistance and allows a holistic approach analyzing different chemical compounds simultaneously. We show that the metabolomes of thrips-resistant and -susceptible hybrids differed considerably. Thrips-resistant hybrids contained higher amounts of the pyrrolizidine alkaloids (PA), jacobine, and jaconine, especially in younger leaves. Also, a flavanoid, kaempferol glucoside, accumulated in the resistant plants. Both PAs and kaempferol are known for their inhibitory effect on herbivores. In resistant and susceptible F_2 hybrids, young leaves showed less thrips damage than old leaves. Consistent with the optimal plant defense theory, young leaves contained increased levels of primary metabolites such as sucrose, raffinose, and stachyose, but also accumulated jacaranone as a secondary plant defense compound. Our results prove NMR as a promising tool to identify different metabolites involved in herbivore resistance. It constitutes a significant advance in the study of plant–insect relationships, providing key information on the implementation of herbivore resistance breeding strategies in plants.

Keywords *Senecio* · Thrips (*Frankliniella occidentalis*) · NMR metabolomics · Pyrrolizidine alkaloids · Flavanoids · Jacaranone

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Introduction

Over the last few decades, western flower thrips (*Frankliniella occidentalis*) has spread worldwide to become a key insect pest of agricultural and horticultural crops (Kirk and Terry 2003). There has been a massive increase in the international movement of plant material leading to the accidental transport of thrips. Thrips have many traits that predispose them to be successful invaders: small size, affinity for enclosed spaces, and high reproductive potential (Lawton and Brown 1986). In addition, western flower thrips is highly polyphagous infesting a wide range of about 200 wild and cultivated host species (Mantel and van de Vrie 1988). Thrips have piercing-sucking mouthparts, which enable them to feed on different types of plant cells (Hunter and Ullman

1989). Feeding on actively growing tissue leads to distortion, reduction in plant growth, and eventually yields loss, while feeding on expanded tissue results in the characteristic silver leaf scars, which affect product appearance and reduce market quality (de Jager et al. 1995a). Western flower thrips causes indirect damage as the primary vector of tospoviruses (Maris et al. 2002).

Occurrence of host plant resistance to thrips is sparse and little is known about the underlying mechanisms. Morphological plant traits were not involved in resistance to western flower thrips in chrysanthemum (de Jager et al. 1995a). Instead, resistance was influenced by the chemical composition of host plants (de Jager et al. 1995b, 1996). Thrips preferentially feed on the older chrysanthemum leaves (de Jager et al. 1995a). No specific metabolites directly related to the resistance have yet been identified. Lately, a novel isobutylamide was suggested to be associated with host plant resistance to western flower thrips in chrysanthemum (Tsao et al. 2005). Overexpression of cysteine protease inhibitors in transgenic chrysanthemum was not related to thrips resistance (Annadana et al. 2002). In contrast, transgenic potato multidomain cysteine protease inhibitors were affiliated with thrips resistance (Outchkourov et al. 2004a, b).

Plant hybridization may drive the evolution of novel secondary plant metabolites (Rieseberg and Ellstrand 1993; Orians 2000) leading to enhanced resistance to herbivores and pathogens (Fritz 1999). In an earlier study of our group the metabolic profiles of *Senecio jacobaea* and *Senecio aquaticus* and their F_1 hybrids revealed multiple putative defense compounds (Kirk et al. 2005). This plant material was used to establish a F_2 hybrid generation to obtain independent segregation of defense characters. In this study, we investigated the differences in metabolites among thrips-resistant and -susceptible hybrids of the F_2 hybrid generation of *S. jacobaea* and *S. aquaticus*. We especially looked at metabolic differences among young and old leaves.

Plants produce a wide array of metabolites which play a significant role in their interactions with other organisms. The study of chemical host plant resistance has so far, for technical reasons, been limited to the identification of single compounds. However, as is generally the case in biological processes, it is very likely that not one but several compounds are involved in plant resistance, the identity of which is, a priori, unknown. One way to get round this problem is to use an analytical tool which allows the simultaneous detection of a wide range of metabolites, providing an instantaneous image of the metabolome of the resistant plant.

Nuclear magnetic resonance spectroscopy (NMR)-based metabolomics may be one of the methods which most fit this description, allowing the simultaneous detection of many different compounds, thus contributing to a deeper holistic approach (Verpoorte et al. 2007). So far, in the field of plant metabolomics, NMR has been successfully applied to study the effect of pathogen infection on host plants such as phytoplasmas in *Catharanthus roseus* (Choi et al. 2004) and tobacco mosaic virus in *Nicotiana tabacum* (Choi et al. 2006). The effect of herbivores on plants has been studied with NMR spectroscopy for chewing insects such as the caterpillars *Plutella xylostella* and *Spodoptora exigua* in *Brassica rapa* (Widarto et al. 2006).

We present the results of three different bioassays intended to identify thrips-resistant *Senecio* F_2 hybrids on which NMR-based metabolomics was subsequently applied to study the metabolic basis of resistance. NMR data were analyzed using diverse multivariate data analyses for comparison. Classification of data was validated by permutation tests.

Methods and Materials

Plant Materials Cuttings of 33 different F_2 hybrids of *S. jacobaea* and *S. aquaticus* derived from tissue culture were transplanted into 11 cm diameter pots filled with equal parts of dune sand and potting soil in spring 2005. Three clonal replicates of each F_2 hybrid were transferred to a growth chamber (18:6 L/D, 20:15°C) and grown for 6 weeks. To obtain resistant and susceptible genotypes, we subjected the 33 F_2 hybrids to a thrips choice bioassay. We then selected the F_2 hybrids with the lowest and the highest thrips damage and confirmed their difference in resistance conducting a non-choice and a dual leaf disc bioassay. Plants of the susceptible and resistant F_2 hybrids for the bioassays as well as for NMR metabolomics were generated from tissue culture and grown as described above.

Thrips Bioassays—Whole Plant Choice Bioassay to Identify the Most Resistant and the Most Susceptible F_2 Hybrids In a fully randomized design, three clonal replicates of each F_2 hybrid were subjected to a thrips infestation with *F. occidentalis*, reared on chrysanthemum. According to van de Wetering et al. (1998), there are no significant differences in leaf area damaged between adult non-viruliferous male and female *F. occidentalis* of different ages. Per plant, ten adult thrips, two males and eight females, were used and left on the plants for 3 weeks. Thrips were directly released into the growth chamber by positioning a vial with 4×10 thrips on plant height in the center of four plants. Thrips does not cause growth damage in *Senecio*; therefore, only silver damage, expressed as the leaf area damaged in square millimeter, was visually scored per plant. The average of the three replicates was used to analyze differences in silver damage among the F_2 hybrids using a one-way

ANOVA (Sokal and Rohlf 1995). Differences in silver damage among the third youngest and the third oldest leaf were tested with a paired samples *t*-test (Sokal and Rohlf 1995). Data of thrips damage were not normally distributed and were, therefore, ln-transformed. To confirm the identification of thrips-resistant F_2 hybrids, we applied two more bioassays: the whole plant non-choice and the leaf disc dual choice bioassay.

Whole Plant Non-choice Bioassay One plant of each of the four most resistant and the four most susceptible F_2 hybrids were placed into individual thrips proof cages, consisting of plastic cylinders (80 cm height, 20 cm diameter), closed on both ends with displaceable rings of thrips proof gauze. The plants were arranged in a fully randomized design and to each cage ten adult western flower thrips were added and left for 3 weeks. Thereafter, silver damage, expressed as the leaf area damaged in square millimeter, was visually scored for each leaf. In order to investigate morphological and mechanical resistance, leaf age, hairiness, toughness, and dry weight were measured. Leaf age was counted in days, twice a week. Leaf hairiness and toughness were measured at the time of scoring for thrips damage. At two locations of each leaf the hairs per square centimeter were counted and the toughness was measured with a penetrometer. Averages per leaf were calculated. Plants were dried for 3 days in an oven at 50°C whereupon plant dry weight was measured for each leaf. Silver damage was not normally distributed. Silver damage per leaf was added up to analyze differences among resistant and susceptible hybrid plants with a Kruskall–Wallis test (Sokal and Rohlf 1995). To study the relationship between silver damage per leaf and leaf age, hairiness, toughness, and dry weight, correlations, using the non-parametric Kendall's tau *b* correlation coefficient, were applied (Sokal and Rohlf 1995).

Leaf Disc Dual Choice Bioassay Dual choice assays were used to test the thrips preference of pairs of leaf discs from resistant versus susceptible F_2 hybrids as described by Outchkourov et al. (2004a). Leaf discs deriving from three different hybrid pairs were used. Pairs were matched at random. For each pair, the bioassay was repeated five times. Two leaf discs of 21 mm in diameter were punched from mature leaves at a similar position and placed on a thin layer of 1% water agar in a 9-cm diameter Petri dish. A piece of filter paper (5×5 mm) was positioned between the discs. Ten female *F. occidentalis*, which had been starved for one night, were shortly anesthetized with CO_2, and placed on the filter paper. The Petri dishes were sealed with parafilm and placed in a growth chamber (16:8, L/D, 20:20°C). The number of thrips on each leaf disc was recorded at different time points after the start of the experiment up to 26 h. As the repeated measurements were not independent, multiple time point measurements were averaged into an early (0–6 h after starting the experiment) and a late period (21–26 h after starting the experiment). Data were analyzed with a one-sided Wilcoxon signed rank test on the absolute differences of average numbers of thrips on the susceptible and resistant leaf discs (Sokal and Rohlf 1995).

Metabolomics—Extraction of Plant Material Ten plants each of the four most resistant and the four most susceptible F_2 hybrids, as identified in the three thrips bioassays, were used for NMR metabolomics. Plants were grown under standard conditions, described above, for 6 weeks. Each third leaf from above (young leaf) and each third leaf from below (old leaf) were taken for analysis. Each sample was ground under liquid N_2 and freeze-dried. Freeze-dried plant material (50 mg) was transferred to a 2-ml microtube. Following a previous method (Choi et al. 2006), 1.5 ml of a mixture of KH_2PO_4 buffer (pH 6.0) in D_2O and methanol-d_4 (1:1) was used for the extraction of the plant samples.

For pyrrolizidine alkaloids extraction, a previously reported method was modified (Pieters et al. 1989). For this extraction, the ten replicates of each of the four thrips-resistant and -susceptible hybrids were combined. Three replicates of this combination were analyzed for each hybrid. An amount of 100 mg dried leaves was ultrasonicated for 30 min with dilute H_2SO_4 (0.5 M). After filtration, the filtrate was adjusted pH 9–10 with 10% ammonium water and fractionated two times with $CHCl_3$. The combined $CHCl_3$ fractions were dried with Na_2SO_4 and evaporated to dryness. The dried extracts were dissolved in $CDCl_3$ containing 0.447 μmol/ml of hexamethyldisiloxane (HMDSO).

NMR Analysis NMR spectra were recorded at 25°C on a 600 MHz Bruker DMX-600 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 600.13 MHz. MeOH-d_4 was used as the internal lock. Each ^{1}H-NMR spectrum consisted of 256 scans requiring 8 min and 30 s acquisition time with the following parameters: 0.12 Hz/point, pulse width (PW) of 30 (11.3 μs), and relaxation delay (RD) of 2 s. A pre-saturation sequence was used to suppress the residual H_2O signal with low power selective irradiation at the H_2O frequency during the recycle delay. Free induction decay was Fourier-transformed with a line broadening factor of 0.3 Hz. The resulting spectra were manually phased and baseline corrected, and calibrated to the internal standard trimethyl silyl propionic acid sodium salt (TMSP) at 0.0 ppm using XWIN NMR (version 3.5, Bruker). The parameters of 2D NMR experiments such as J-resolved, COSY, HSQC, and HMBC were the same as those of our previous reports (Choi et al. 2006).

Table 1 Silver damage (square millimeter) of the four most resistant and the four most susceptible *Senecio* F_2 hybrid lines

	Hybrid	Silver damage
Resistant	217	8±5
	115	12±5
	119	16±6
	206	37±18
Susceptible	108	275±80
	204	288±132
	221	307±60
	114	349±167

Data are means and standard errors of three replicates

For 1D and 2D NMR analysis of pyrrolizidine alkaloids, the same parameters were used without pre-saturation of water. Calibration was performed on residual $CDCl_3$ signal at δ 7.26.

Data Analysis Spectral intensities of ^{1}H-NMR spectra were scaled to the intensity of the internal standard (TMSP, 0.05% *w*/*v*) and reduced to integrated regions of equal width (0.04) corresponding to the region of δ 0.4–δ 10.0. The regions of δ 4.8–δ 4.9 and δ 3.28–δ 3.40 were excluded from the analysis because of the residual signal of water and MeOH. Principal component analysis (PCA) and partial least square-discrimination analysis (PLS-DA) were performed with the SIMCA-P software (v. 11.0, Umetrics, Umeå, Sweden). The scaling method for PCA was Pareto and for PLS-DA the unit-variance method. ANOVA, hierarchical clustering analysis, and *t* tests were performed by MultiExperiment Viewer (v. 4.0; Saeed et al. 2003). PLS-DA models were validated by using the permutation method through 20 applications, which is a default validation tool in the software package applied (SIMCA-P). Variance (R^2) and cross-validated variance values (predictive ability of the model, Q^2) of PLS-DA using five components were calculated.

Results

Whole Plant Choice Bioassay The F_2 hybrids differed significantly in thrips damage (ANOVA, F=3.17, df=32, P<0.001). In the most resistant hybrid, an 8 mm^2 leaf area was affected by silver damage compared to 349 mm^2 in the most susceptible hybrid. Young leaves had significantly less silver damage compared to mature leaves (*t*-test, t=5.87, df=32, P<0.001). Of these 33 F_2 hybrids, the four most resistant and the four most susceptible ones (Table 1) were chosen for further testing in a whole plant non-choice and a leaf disc dual choice bioassay.

Whole Plant Non-choice Bioassay The four thrips-resistant F_2 hybrids, described in the above whole plant choice bioassay, had significantly (Kruskall–Wallis test, χ^2=5.33, df=1, P=0.029) less thrips damage per plant (94 mm^2) compared to the four susceptible hybrids (368 mm^2). The results of the plant choice and the non-choice bioassay were thus completely compatible. Resistant F_2 hybrids had on average 15 and susceptible ones 13 leaves, respectively (*t*-test, t=1.55, df=6, P=0.17). Silver damage was for all four resistant and all four susceptible F_2 hybrids significantly

Table 2 Correlations (Kendall's tau) between silver damage (square millimeter) per leaf and morphological (leaf age) as well as mechanical (toughness, hairiness, and dry weight) resistance factors of four thrips-resistant and four thrips-susceptible *Senecio* F_2 hybrid lines

	Hybrid	Leaf age	Toughness	Hairiness	Dry weight
Resistant	217	0.734	−0.354	−0.743	0.559
	N=13	P=0.003**	P=0.167	P=0.003**	P=0.022*
	115	0.716	0.279	−0.750	0.716
	N=10	P=0.007**	P=0.321	P=0.005**	P=0.007**
	119	0.552	−0.327	−0.331	0.378
	N=15	P=0.008**	P=0.162	P=0.115	P=0.072
	206	0.597	−0.166	−0.597	0.464
	N=14	P=0.011*	P=0.519	P=0.011*	P=0.048*
Susceptible	114	0.671	−0.379	−0.739	0.648
	N=14	P=0.001**	P=0.075	P=0.000***	P=0.002**
	221	0.726	0.322	−0.658	0.393
	N=14	P=0.002**	P=0.188	P=0.005**	P=0.093
	204	0.664	−0.252	−0.503	0.554
	N=16	P=0.002**	P=0.249	P=0.021*	P=0.008**
	108	0.486	−0.039	−0.299	0.505
	N=15	P=0.014*	P=0.854	P=0.132	P=0.011*

N indicates the number of leaves the correlation is based on

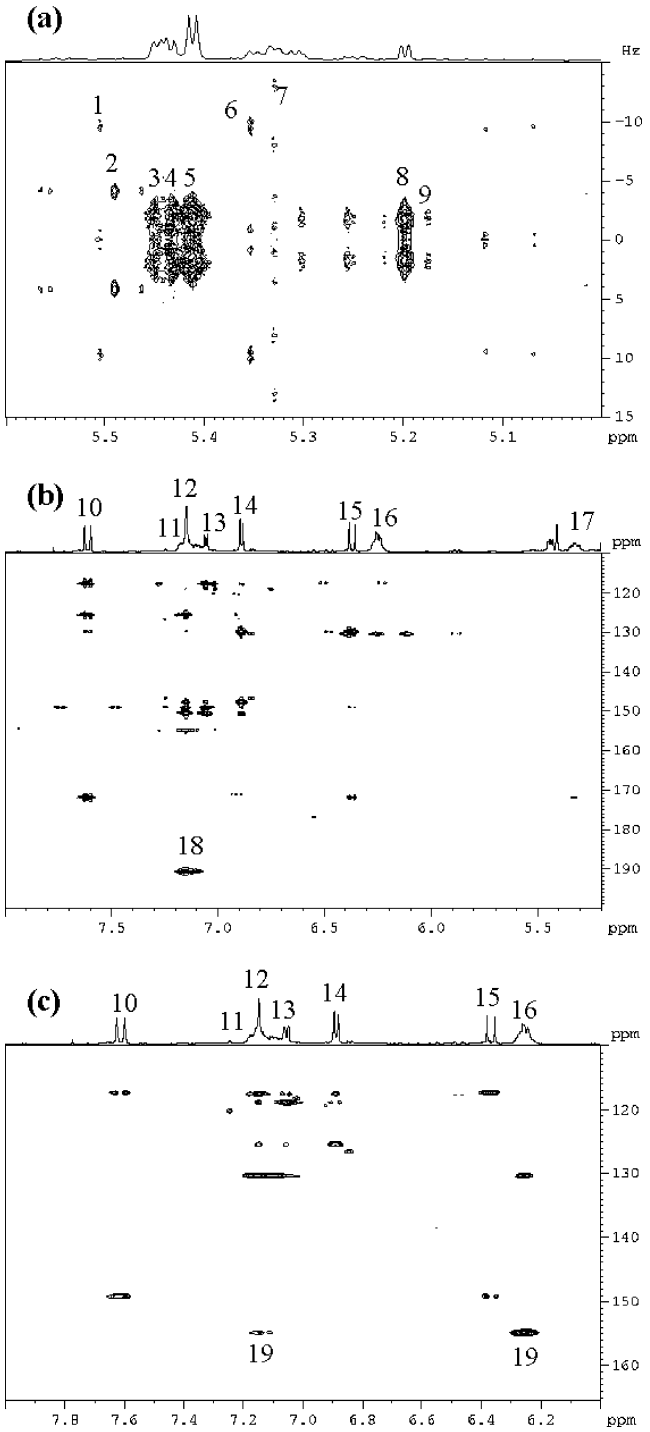


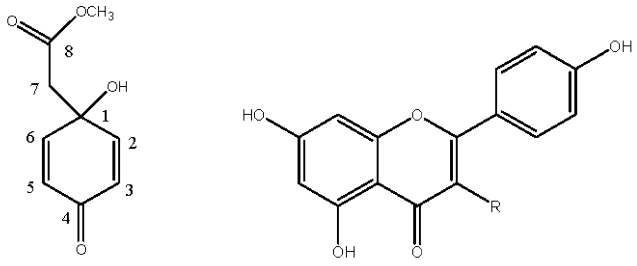
Fig. 1 Typical 2D J-resolved NMR spectrum (**a**), HMBC (**b**), and HSQC–TOCSY (**c**) of *Senecio* F_2 hybrids. *1*, H-3 of feruloyl quinic acid; *2*, H-1′ of kaempferol glucoside; *3* H-1′ of stachynose; *4*, H-1′ of raffinose; *5*, H-1 of sucrose; *6*, H-3 of 3-*O*-caffeoyl quinic acid; *7*, H-5 of chlorogenic acid; *8*, H-1 of α-glucose; *9*, H-1 of trehalose; *10*, H-7 of chlorogenic acid; *11*, H-3 and H-5 of jacaranone; *12*, H-2′ of chlorogenic acid; *13*, H-6′ of chlorogenic acid; *14*, H-5′ of chlorogenic acid; *15*, H-8′ of chlorogenic acid; *16*, H-2 and H-6 of jacaranone; *17*, H-5 of chlorogenic acid; *18*, correlation between H-3, H-5, and C-4 of jacaranone; *19*, correlation between H-2, H-3, H-5, H-6, and C-1 of jacaranone

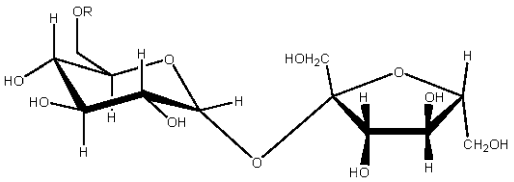
positively correlated to leaf age (Table 2). None of the morphological and mechanical factors was related to thrips resistance.

Leaf Disc Dual Choice Bioassay In all three pairs of leaf discs of resistant versus susceptible F_2 hybrids, the number of thrips in the early (0–6 h after start of the experiment) period was significantly lower on the leaf disc of the resistant hybrid (Wilcoxon signed rank test, P=0.028; 0.042; 0.018). For two out of the three leaf pairs, this was true for the late period (21–26 h after starting the experiment) as well (Wilcoxon signed rank test, P=0.043; 0.046; 0.715).

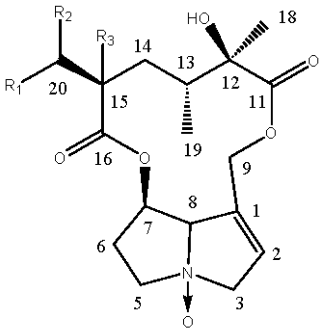
Application of the 1D and 2D NMR Spectroscopy for Elucidation of Metabolites The ^{1}H-NMR spectra of plant extracts always give congested signals. In our previous work, 2D J-resolved spectra were successfully applied to overcome the signal overlapping (Choi et al. 2006). Indeed, by applying J-resolved spectra in this study, the signals of flavanoid glycosides, raffinose, stachyose, and sucrose were clearly resolved (Fig. 1a). In the aromatic region, two isomeric phenylpropanoids, 3-*O*-caffeoyl quinic acid and 5-*O*-caffeoyl quinic acid (chlorogenic acid), as well as feruloyl quinic acid, were identified using 2D NMR spectra including COSY, HSQC, and HMBC. These metabolites were confirmed by comparison with reference compounds and reported data (Choi et al. 2006). In addition to phenylpropanoids, there were two major signals at δ 6.25 and δ 7.15, which correlated with each other in the COSY spectrum. Using HMBC and HSQC–TOCSY spectra, those two signals at δ 6.25 and δ 7.15 were assigned as H-2 and H-6, and H-3 and H-5 of jacaranone, respectively (Fig. 1b, c). The characteristic chemical shifts and the structures of the metabolites of *Senecio* F_2 hybrid leaves detected in the ^{1}H-NMR spectra are shown in Fig. 2 and Table S1.

Principal Component Analysis and Partial Least Square Regression-Discriminant Analysis of ^{1}H-NMR Data Among multivariate data analyses, the most common unsupervised method is principal component analysis (PCA). Thus, as the first step of multivariate data analysis PCA was performed on the bucketed dataset to discriminate the samples. For the dataset obtained from the ^{1}H-NMR analysis, a 22-component model explained 98.5% of the variance, with the first two components explaining 70.7%. However, as shown in Fig. 3a, there was no separation among resistant and susceptible *Senecio* F_2 hybrids. Instead, developmental stage was a key separating factor that led to a clear discrimination among young and old leaves. According to the loading plot, the levels of citric and malic acid were higher in old leaves whereas those of

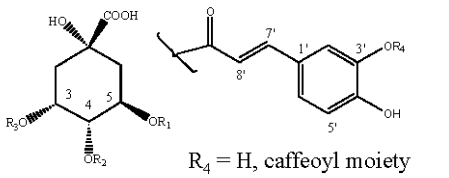




R = H, sucrose
R = α-D-galactopyranosyl (1→6), raffinose
R = α-D-galactopyranosyl (1→6), α-D-galactopyranosyl (1→6), stachyose



R_1 = H, R_2 = R_2 = —O—, jacobine-*N*-Oxide
R_1 = Cl, R_2 = H, R_3 = OH, jaconine-*N*-Oxide



R_4 = H, caffeoyl moiety
R_4 = OCH_3, feruloyl moiety

R_1 = caffeoyl, R_2 = R_3 = H, chlorogenic acid
R_1 = H, R_2 = H, R_3 = caffeoyl, 3-*O*-caffeoylquinic acid
R_1 = H, R_2 = H, R_3 = feruloyl, 3-*O*-feruloyllquinic acid

Fig. 2 Chemical structures of some metabolites identified in the NMR spectrum of *Senecio* F_2 hybrids

sucrose, raffinose, and stachyose as well as jacaranone accumulated more in young leaves (Fig. 3b).

In the dataset employed, the difference among resistant and susceptible leaves seemed to be smaller than among developmental stages. The large metabolic difference in developmental stages compared to the difference among resistant and susceptible plants was confirmed by hierarchical analysis (HCA, Fig. S1). Prior to HCA, the statistically significant signals were selected applying ANOVA. From 239 ^{1}H-NMR signals 161 were significant (P<0.05). The ANOVA was based on one factor containing four groups: resistant young leaves, susceptible young leaves, resistant old leaves, and susceptible old leaves. Two additional sets of t tests, one for young and one for old leaves comparing resistant and susceptible plants were conducted (P<0.05). These tests showed that for young leaves, 56 signals out of 239, and for old leaves, 25 signals out of 239, were related to thrips resistance. In HCA, the leaves of the same age were closely clustered but the metabolic resemblance within resistant or susceptible plants was scattered. In order to distinguish resistant leaves from susceptible ones, analysis was extended to PLS-DA, a supervised multivariate data technique.

PLS-DA uses a discrete class matrix (0 and 1) as an additional matrix. In contrast to PCA that only uses the information of the metabolomic matrix, PLS-DA also takes into account the resistance matrix. The separation of PLS-DA is achieved by the covariance of the two datasets. When PLS-DA was applied the separation of thrips-resistant and -susceptible plants considerably improved (Fig. 4a). Validation of the PLS-DA model by permutation tests resulted in a variance R^2 of 0.74 and a cross-validated variance Q^2 of 0.61. All Q^2 values of the permuted Y vectors were lower than the original ones and the regression of Q^2 lines intersected at below zero (Fig. 4c, d).

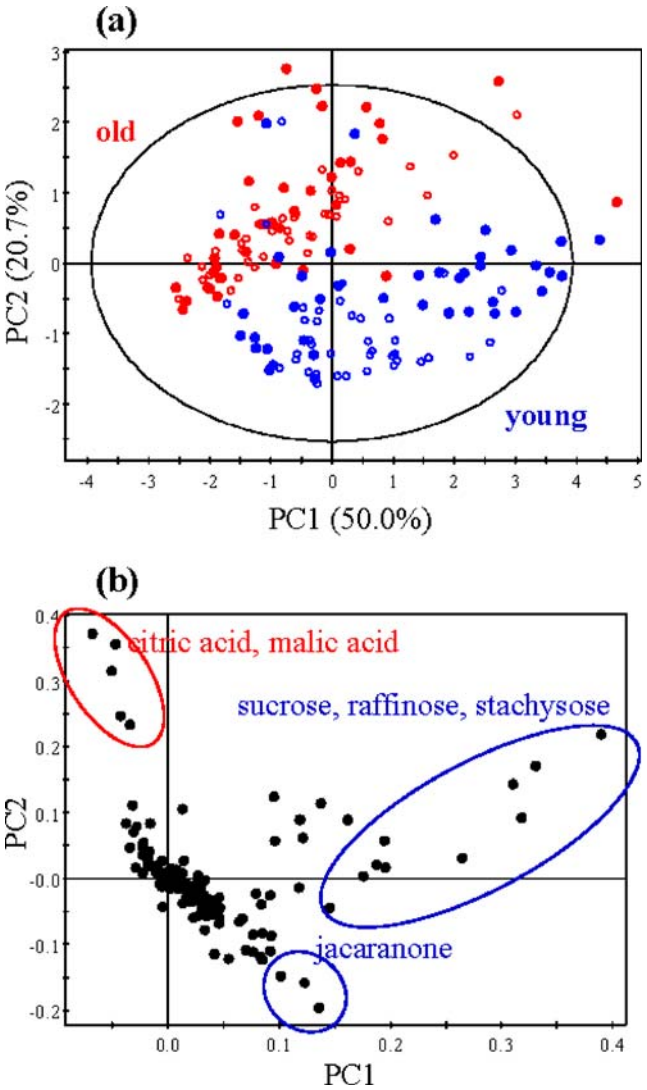


Fig. 3 Score (**a**) and loading plot (**b**) of principal component analysis based on ^{1}H-NMR spectra of *Senecio* F_2 hybrid genotypes. *Filled red circle*, old leaves of thrips-resistant genotypes (n=40); *filled blue circle*, young leaves thrips-resistant genotypes (n=40); *empty red circle*, old leaves of thrips-susceptible genotypes (n=40); *empty blue circle*, young leaves of thrips-susceptible genotypes (n=40). The *ellipse* represents the Hotelling T2 with 95% confidence in score plots

Irrespective of leaf age, resistant and susceptible F_2 hybrids were clearly clustered. The loading plot indicated that methyl signals of pyrrolizidine alkaloids (PAs) at δ 1.12 (H-19), δ 1.24 (H-21), and δ 1.36 (H-18; Pieters et al. 1989) were distinguishably higher in the resistant plants. Also, the anomeric proton of kaempferol glucoside at δ 5.50–δ 5.60 together with H-6 and H-8 of the glycoside around at δ 6.30 increased in the *Senecio* F_2 hybrids resistant to thrips (Fig. 4b). Only total PA could be determined by the methyl signals detected. Therefore, more evidence for the PAs, e.g., the signals of pyrrolizidine moiety, was required to identify specific compounds. The characteristic H-2 signal of the pyrrolizidine moiety is generally detected around at δ 6.0–δ 6.2. However, this region overlaps with the signals of jacaranone and chlorogenic acid. For the confirmation and quantification of individual PAs, we applied an acid–base extraction followed by NMR metabolomic profiling. For the acid–base extraction the ten replicates of each of the four thrips-resistant and -susceptible hybrids were mixed and three replicates of these used for the analysis.

Elucidation and Quantitation of Pyrrolizidine Alkaloids Using Acid–Base Extraction Followed by Quantitative ^{1}H-NMR The ^{1}H-NMR spectrum of the alkaloid extract, obtained by the acid–base extraction, showed two major PAs, jacobine and jaconine N-oxide. The N-oxides were confirmed by the unambiguous down field shifts (0.4–0.7 ppm) of the signals of H-3, H-5, and H-8 adjacent to the N-oxide functional group when compared with the free bases (Pieters et al. 1989; Witte et al. 1992). For the final structure confirmation, COSY, TOCSY, HSQC, and HMBC spectra were employed (Fig. S2).

The ^{1}H-NMR signals are always proportional to the molar concentrations. In the crude alkaloid extract, H-2 was detected in a non-crowded region, separated from other ones. These signals were used for quantification comparing them to the intensity of the internal standard, HMDSO (0.48 μmol/ml). As confirmed by the PLS-DA results, young and old leaves of thrips-resistant F_2 hybrids accumulated more PA N-oxide (Fig. 5). Old (t-test, df=22, P<0.001) and young (t-test, df=22, P<0.001) leaves of thrips-resistant hybrids contained significantly more jacobine N-oxide. Younger leaves of thrips-resistant hybrids also accumulated significantly more jaconine N-oxide (t-test, df=22, P=0.005). The difference of jaconine N-oxide in old leaves was close to significance (t-test, df=22, P=0.008). Young leaves in both resistant and susceptible hybrids accumulated more jacobine N-oxide (resistant hybrids: t-test, df=22, P<0.001; susceptible hybrids: t-test, df=22, P<0.001) and more jaconine N-oxide (resistant hybrids: t-test, df=22, P<0.001; susceptible hybrids: t-test, df=22, P<0.001).

Discussion

NMR proved to be successful in the simultaneous detection of different metabolites potentially involved in thrips resistance. The metabolomic profiles of thrips-resistant and -susceptible hybrids differed considerably. Thrips-resistant F_2 hybrids showed significantly higher amounts of the PAs, jaconine and jacobine N-oxide, and a flavanoid, kaempferol glucoside.

PAs have been well studied in the *Senecio* species and are regarded as constitutive defense against generalist

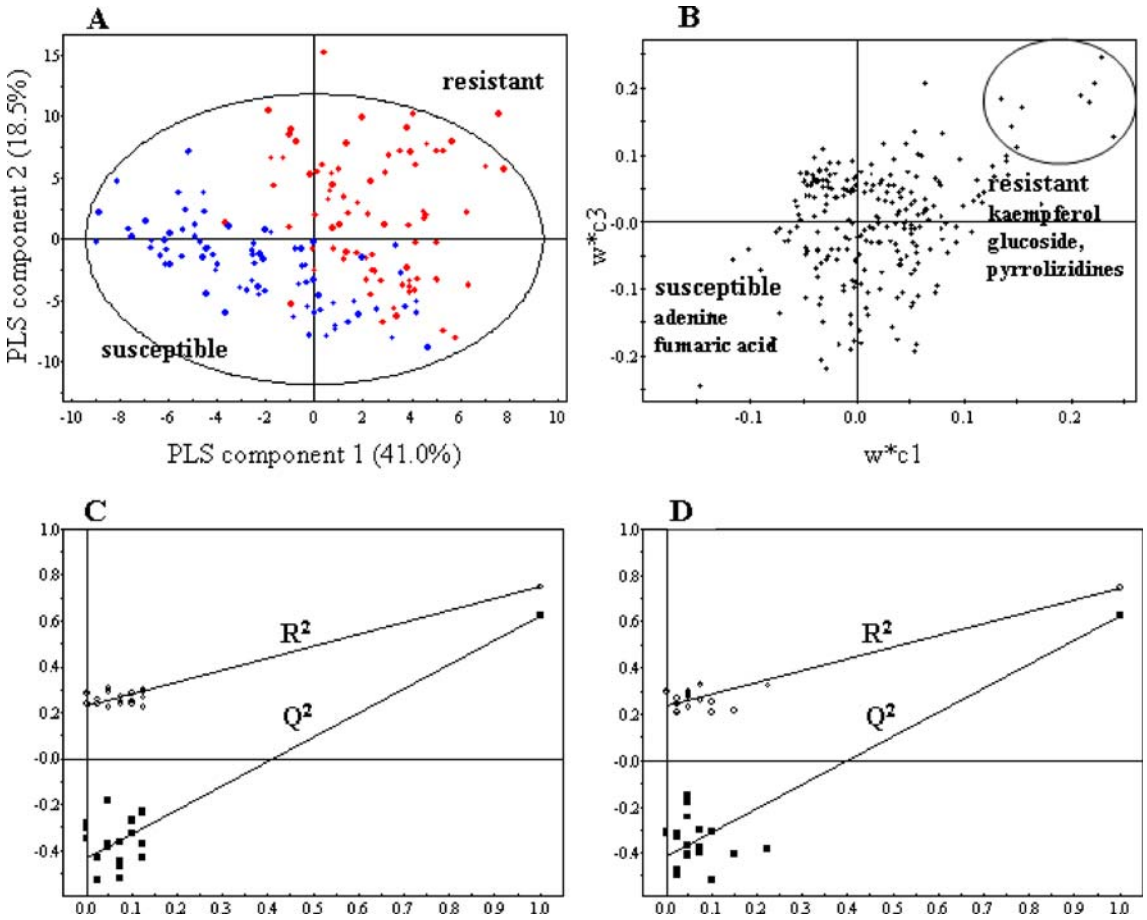


Fig. 4 Score (**a**), loading plot (**b**), and permutation validation plot of resistant (**c**) and susceptible group (**d**) of partial least square regression-discriminant analysis based on ^{1}H-NMR spectra of *Senecio* F_2 hybrid genotypes. *Filled red circle*, old and young leaves of thrips-resistant genotypes (n=80); *filled blue circle*, old and young leaves thrips-susceptible genotypes (n=80). The *ellipse* represents the Hotelling T2 with 95% confidence in score plots

herbivores. They deter chewing insects such as caterpillars (Bentley et al. 1984; van Dam et al. 1995), locusts (Macel et al. 2005), and beetles (Hägele and Rowell-Rahier 2000). PAs also have a negative effect on sucking insects such as aphids (Vrieling et al. 1991; Macel et al. 2005). So far, no inhibitory influences of PAs on thrips have been detected. Plants of *S. jacobaea* with and without *Haplothrips senecionis*, a specialist on *Senecio* species, did not yield any difference in total PA concentration (Vrieling et al. 1991). In vitro tests studying the effect of different PAs on the development of first instar larvae of the generalist *F. occidentalis* showed a significantly reduced larval survival at 10 × plant concentrations but not at 1 × plant concentrations (Macel et al. 2005). Neither jaconine nor jacobine were included in these in vitro tests. However, a significantly lower amount of thrips damage on the jacobine compared to the erucifoline chemotype of *S. jacobaea* (Macel 2003) confirms our results. Furthermore, PAs are stored in the cell vacuoles (Ehmke et al. 1988), which are ingested by the thrips being cell feeders. Both PAs, jaconine and jacobine, were present in higher concentrations in young leaves compared to the old ones. Concurrently, young leaves showed significantly less thrips damage. This is in accordance with the finding that in *Senecio* species, young leaves have relatively high concentrations of PAs compared to old leaves (Hartmann and Zimmer 1986; de Boer 1999). The more valuable plant parts are better defended against generalist herbivores than less important organs as is predicted by the optimal defense theory (Zangerl and Bazzaz 1992; van Dam et al. 1996).

Flavanoids are generally involved in plant resistance to herbivores (Bennett and Wallsgrove 1994). The presence of kaempferol in the *Senecio* F_2 hybrids confirms the findings of Kirk et al. (2005) reporting kaempferol for the first time in *Senecio* species. Kaempferol is known to convey a deterrent effect on generalist caterpillars (Onyilagha et al. 2004). Aphid resistant cow pea lines contained significantly higher amounts of flavanoids, including kaempferol, compared to

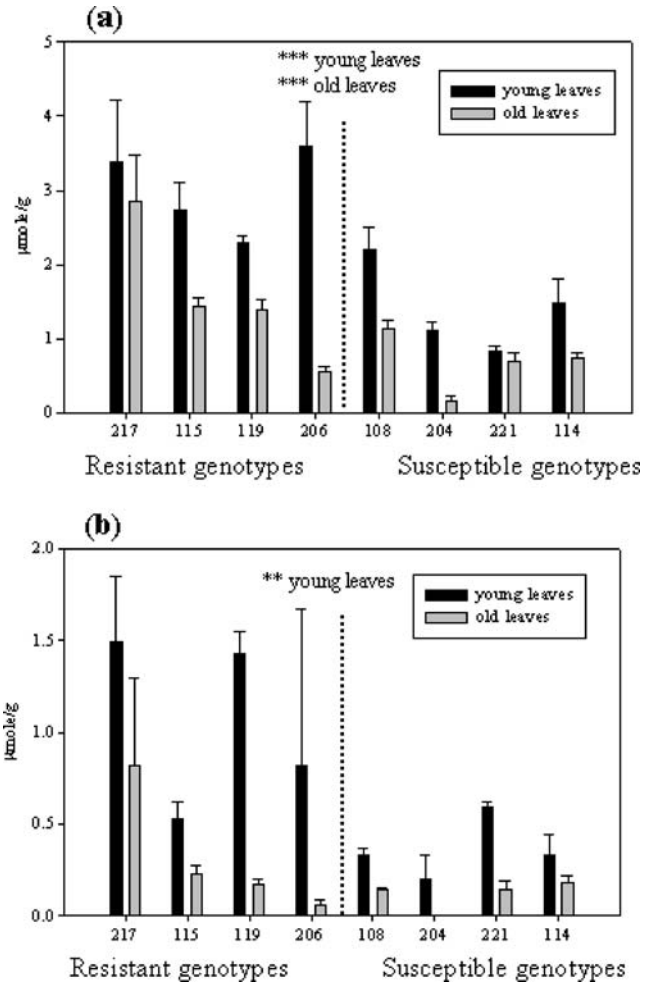


Fig. 5 Yields (mol/g) of jacobine N-oxide (**a**) and jaconine N-oxide (**b**) for young and old leaves of thrips-resistant and -susceptible *Senecio* F_2 hybrids obtained by quantitative ^{1}H-NMR analysis. Data are based on the combination of the ten replicates of each of four thrips-resistant and -susceptible hybrids, of which three replicates were analyzed per hybrid. Means and their standard deviation are presented. Data were analyzed by *t* tests. Significant differences in PAs between thrips-resistant and -susceptible hybrids are designated by ***$P<0.001$ and **$P<0.01$. Young leaves contained significantly higher amounts of both PAs in resistant and susceptible hybrids (all *t* tests $df=22$, $P<0.001$)

susceptible lines (Lattanzio et al. 2000). Recently, kaempferol has been identified to confer resistance to onion thrips (*Thrips palmi*) in golden rod (*Solidago altissima*; Wu et al. 2007). Like western flower thrips, onion thrips is a polyphagous worldwide pest, which causes significant economic losses (Kirk and Terry 2003).

Besides the PAs and flavanoids, specifically involved in thrips resistance, three other phenolic compounds involved in plant–herbivore interactions, 3-*O*-caffeoyl acid and 5-*O*-caffeoyl quinic acid (chlorogenic acid, CGA), and feruloyl quinic acid (FQA), were identified to be present in the metabolome of the *Senecio* F_2 hybrids. CGA has been described as an anti-feedant and digestibility reducer in different caterpillar species (Mallikarjuna et al. 2004; Johnson and Felton 2001; Simmonds and Stevenson 2001) and sucking insects such as aphids (Miles and Oertli 1993). FQA has been implicated in the resistance of sucking herbivores such as cereal aphids (Cabrera et al. 1995; Havlickova et al. 1996) and cereal midges (Ding et al. 2000; Abdel-Aal et al. 2001). FQA is a precursor of lignin conferring rigidity to cell walls (Bennett and Wallsgrove 1994). As such, it is linked to the resistance against stem borers in maize (Santiago et al. 2006; Mao et al. 2007) and cotton (Wang et al. 2006).

As expected, the young leaves of the *Senecio* F_2 hybrids, being photosynthetically more active, contained more primary metabolites such as sugars, compared to older leaves. They are, therefore, more attractive to herbivores and pathogens. To defend these valuable plant parts, they do contain more secondary metabolites related to plant defense. Next to the increased amount of PAs in the young leaves of the thrips-resistant *Senecio* F_2 hybrids, young leaves in general contained more jacaranone. Analogues of jacaranone are known to occur in different *Senecio* species (Lajide et al. 1996; Xu et al. 2003; Kirk et al. 2005). They have shown insecticidal activity against houseflies (Xu et al. 2003) and inhibited growth in the generalist herbivore *Spodoptera litturalis* (Lajide et al. 1996).

Our approach to apply NMR as a technique to identify metabolites differing between thrips-resistant and -susceptible plants proved to be very successful. NMR indicated three different metabolites as potential candidates for thrips resistance. While the PAs due their toxic effect on mammals (Cheeke 1988) may be interesting in developing thrips resistance in ornamentals, kaempferol and jacaranone are very promising candidates to develop host plant resistance to thrips in crops. Not only do they inhibit thrips but they also produce positive effects on human health. Jacaranone has been investigated as a potential anti-cancer agent (Loizzo et al. 2007). Flavanoids in general caused a significant extension of life span in cancer-susceptible mice (Butelli et al. 2008), while kaempferol in particular demonstrated a cytotoxic activity on human cancer cell lines (Daniela et al. 2007; Li et al. 2007). A tomato with increased amounts of flavanoids, whereby kaempferol glycoside accounted for 60% of this increase, has already been engineered (Le Gall et al. 2003).

Applying NMR to the study of herbivore resistance constitutes a significant advance in the study of plant–insect relationships, providing key information on the implementation of herbivore resistance breeding strategies in plants.

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(*S*)-2-Acetoxy-5-Undecanone, Female Sex Pheromone of the Raspberry Cane Midge, *Resseliella theobaldi* (Barnes)

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Abstract The raspberry cane midge, *Resseliella theobaldi*, is a widespread pest of cultivated red raspberry in Europe. Pheromone-baited traps could provide a much-needed, accurate means to monitor the pest. Volatiles collected separately from virgin female and male midges were analyzed by gas chromatography (GC) coupled to mass spectrometry (MS) to reveal four female-specific components. In analyses by GC coupled to electroantennographic (EAG) recording from the antennae of a male midge, at least three of these components elicited responses. Based on its GC retention indices and mass spectrum, we propose that the major component is 2-acetoxy-5-undecanone and confirm this by synthesis of the racemic compound in seven steps and 63% yield from 4-pentenoic acid. The three minor components were each present at approximately 30% of the major component and were identified as 2-undecanone, (*S*)-2-acetoxyundecane, and (*S*)-2-undecanol by comparison of GC retention times and mass spectra with those of synthetic standards. GC analyses of the female-produced volatiles on an enantioselective column showed that only one enantiomer of 2-acetoxy-5-undecanone was present, and this was found to be the *S*-enantiomer by hydrolytic kinetic resolution of an epoxide intermediate in the synthesis and also by enantioselective hydrolysis of the racemic acetate with a lipase enzyme. The two enantiomers were also separated by high-performance liquid chromatography on an enantioselective column for field tests. In two field trapping tests, (*S*)-2-acetoxy-5-undecanone was highly attractive to male *R. theobaldi*; the *R*-enantiomer was not attractive. The racemic compound was just as attractive as the *S*-enantiomer, and addition of the three minor components in racemic form at two different loads did not affect catches. The pheromone could be dispensed from both rubber septa and polyethylene vials for at least 1 month under field conditions, but the former was preferred as it gave more uniform release. 2-Acetoxy-5-undecanone belongs to a new group of pheromone structures in the Cecidomyiidae, most others being mono- or diesters.

Keywords 2-Acetoxyundecane · 2-Acetoxy-5-undecanone · Cecidomyiidae · Enantioselectivity · Pheromone trap · Raspberry cane midge · *Resseliella theobaldi* · Sex pheromone · 2-Undecanol · 2-Undecanone

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Introduction

The raspberry cane midge, *Resseliella theobaldi* Barnes (Diptera: Cecidomyiidae), is a widespread pest of cultivated

red raspberry, *Rubus idaeus*, and occasionally loganberry, in Europe, both in field-grown crops and under protected cultivation. *R. theobaldi* overwinters as larvae in small earthen cocoons at the base of the plants. After pupation in spring, adults emerge and oviposit in natural splits or wounds in the bark of primocanes. Larvae feed on the newly exposed periderm layer causing penetrating lesions. These feeding sites are colonized by a range of pathogenic fungi, such as the cane blight fungus, *Leptosphaeria coniothyrium*, causing a disease complex known as "midge blight." Attacked canes may snap off, grow poorly, or die. Yield losses frequently exceed 50%, often making the crop unprofitable to harvest. Depending upon latitude, there are three or four generations per year (Cross et al. 2009a).

The pest is currently controlled by scheduled spraying of broad-spectrum insecticides. In the UK, the organophosphate (OP) insecticide chlorpyrifos is applied to control the first generation of larvae in the spring. This also prevents significant damage by subsequent generations, although population increase almost certainly occurs. In other countries where OP insecticides are not available for use on raspberry, synthetic pyrethroid insecticides are used, but these are less effective. Timing of sprays is critical. Traditionally, a spray was applied in late April or early May when the primocane was 20–30 cm high, with a second spray about 2 weeks later. More recently, a temperature-based forecasting model was developed by Gordon et al. (1989) for predicting spring oviposition by *R. theobaldi* to facilitate timing of sprays. The forecast is believed to be accurate to within 5 days, but it nevertheless results in recurring use of insecticides in most commercial plantations. A more locally based, specific monitoring system such as that based on pheromone traps would be preferable.

Many midge species use sex pheromones for mate location (Harris and Foster 1999). The chemical structures of the components of the female-produced sex pheromones have been identified from several species including Hessian fly, *Mayetolia destructor* (Foster et al. 1991), pea midge, *Contarinia pisi* (Hillbur et al. 1999, 2000, 2001), orange wheat blossom midge, *Sitodiplosis mosellana* (Gries et al. 2000), Douglas-fir cone gall midge, *Contarinia oregonensis* (Gries et al. 2002), aphidophagous gall midge, *Aphidoletes aphidimyza* (Choi et al. 2004), and swede midge, *Contarinia nasturtii* (Hillbur et al. 2005). The chemical structures of these pheromones are related to each other, having carbon chains with an odd number of carbon atoms and one or two ester functionalities.

The existence of a female sex pheromone in *R. theobaldi* has not been demonstrated, but it was assumed that one existed by analogy with other species. As part of a program to develop new tools for monitoring and control of this pest, we report identification of components of the female sex pheromone, and we demonstrate that the major component is attractive to conspecific males in the field. This pheromone component is a ketoester and provides a second example among midge pheromone components of this novel type of structure, first discovered by us in the apple leaf curling midge, *Dasineura mali* (Cross and Hall 2005, 2009; Hall et al. 2005; Suckling et al. 2007; Cross et al. 2009b).

Methods and Materials

Collection and Rearing of Midges Mature larvae of *R. theobaldi* were collected on 27 and 28 July 2004 from two infested commercial raspberry plantations of the primocane varieties Autumn Bliss and Joan Squire at Beech Farm, West Peckham, Kent, UK. Sections of primocane of up to 25-cm length with splits and characteristic patch lesions caused by the midge were cut from the canes and stored in ventilated plastic boxes (19×10.6×7.5 cm) with a layer of moistened paper towel in the base. The boxes each contained about 20 cane sections and were kept on the laboratory bench. Many of the mature larvae exited the lesions underneath the epidermis seeking sites to pupate on the side, the lid, or in the base of the boxes, or they pupated in situ. Over a period of 16 days, these mature larvae were collected and transferred individually to small transparent closed Perspex tubes (autoanalyzer cups with caps, internal volume 2 ml; Sarstedt, Leicester, UK). A small piece of moist filter paper was included in each tube to prevent desiccation. The tubes were held on plastic trays in incubators at approximately 18°C and 16:8 h L/D. Adult midges started to emerge on 16 August, and the newly emerged, virgin adults were collected, sexed, and transferred to the entrainment apparatus each day.

Collection of Volatiles from Virgin Midges Volatiles were collected from virgin female and virgin male *R. theobaldi* by placing the two sexes in separate, specially constructed glass containers with a glass frit at the upwind end (15×5 cm; Hamilton Laboratory Glass, Margate, Kent, UK). Air was drawn with a vacuum pump (M361C, Charles Austen Pump, Byfleet, Surrey, UK) into each vessel through a filter of activated charcoal (20×2 cm; 10–18 mesh, Fisher Chemicals, UK) and out through a collection filter consisting of a Pasteur pipette (4 mm i.d.) containing Porapak Q (200 mg, 50–80 mesh; Waters Associates, Milford, MA, USA) held between plugs of silanized glass wool. The Porapak was Soxhlet extracted with chloroform for 8 h, and the filters were washed with dichloromethane immediately prior to use. A low airflow rate (200 ml/min) was used during volatile collection to minimize desiccation stress to the insects. Calling females stood motionless on the sides of the glass vessel with their ovipositors extended to expose

the pheromone gland at the base from which the pheromone was emitted. Male midges flew around erratically. Adult *R. theobaldi* have a short life of only 1–2 days, and dead midges were removed and fresh live ones introduced daily. Porapak filters were changed approximately every week. Over a 3-week period, five collections were made from a total of 987 female midges and five collections from 633 males. Trapped volatiles were eluted with dichloromethane (pesticide grade; 3×0.5 ml), and the resulting solutions were stored at −20°C.

Analysis by Gas Chromatography Gas chromatography (GC) analyses were carried out on a fused-silica capillary column (30 m×0.32 mm i.d.×0.25 μm film thickness) coated with a polar stationary phase (Wax10; Supelco, Gillingham, Dorset, UK). Injection was splitless (220°C), detection was by flame ionization detection (FID; 250°C), and carrier gas was helium (2.4 ml/min). The oven temperature was programmed from 60°C for 2 min, then at 10°C/min to 250°C. Retention times are expressed as retention indices relative to the retention times of normal, saturated hydrocarbons.

Enantioselective GC analyses were carried out on a capillary column (25 mm×0.32 mm i.d.×0.25 μm film thickness) coated with a cyclodextrin stationary phase (Chirasil-DEX CB; Varian, Oxford, UK). Injection was splitless (220°C), detection was by FID (250°C), and carrier gas was helium (2.4 ml/min). For analysis of crude volatile collections, the oven temperature was programmed at 60°C for 2 min, then at 6°C/min to 200°C. For analysis of synthetic compounds, split injection was used, and the temperature program was isothermal (140°C).

Analysis by Gas Chromatography Linked to Mass Spectrometry Gas chromatography-mass spectrometry (GC-MS) analyses were carried out with a Carlo Erba 5130 GC (Thermoelectron, Hemel Hempstead, Hertfordshire, UK) linked directly to an ion trap detector (Finnigan ITD 700; Thermoelectron) operated in electron impact (EI) or chemical ionization with isobutane (CI) modes. The GC column (30 m×0.25 mm i.d.×0.25 μm film thickness) was coated with polar Wax10 (Supelco). The carrier gas was helium (0.5 kg/cm^2), and the oven temperature was programmed from 50°C for 2 min, then at 6°C/min to 250°C.

GC-MS analyses were also carried out on an HP 6890 GC (Agilent) coupled directly to an HP 5973 MSD (Agilent) operated in EI mode. The GC column (30 m× 0.25 mm i.d.×0.25 μm film thickness) was coated with nonpolar SPB1 (Supelco). The carrier gas was helium (1 ml/min), and the oven temperature was programmed from 60°C for 2 min, then at 6°C/min to 250°C.

Analysis by Gas Chromatography Linked to Electroantennography Gas chromatography-electroantennography (GC-EAG) analyses were carried out with an HP 6890 GC (Agilent) fitted with capillary GC columns (30 mm× 0.32 mm i.d.×0.25 μm film thickness) coated with polar (Wax10; Supelco) and nonpolar (SPB1; Supelco) phases. Injection was splitless (220°C), and detection was by FID (250°C). The oven temperature was programmed from 50°C for 2 min, then at 10°C/min to 250°C. The GC column effluents were combined and then split (1:1) between the FID and a silanized glass T-piece in the column oven. Nitrogen (200 ml/min) was blown continuously over the EAG preparation, and every 17 s, this was diverted through the T-piece for 3 s, blowing the contents over the EAG preparation, as described by Cork et al. (1990).

Following experience from working with other midge species, the EAG preparation was set up by suspending the whole insect between glass electrodes containing electrolyte (0.1 M potassium chloride with 10% polyvinylpyrrolidone added to reduce evaporation). The ends of both antennae were inserted into the recording electrode and the body into the reference electrode. The electrodes were inserted onto silver/silver chloride electrodes held in micromanipulators on the portable EAG device developed by Syntech (INR-02; Syntech, Hilversum, The Netherlands). Both FID and EAG signals were collected and analyzed with EZChrom software (Elite v3.0; Scientific Software, Pleasanton, CA, USA).

Synthetic Chemicals 2-Undecanone and racemic 2-undecanol were purchased from Sigma-Aldrich (Gillingham, Dorset, UK). Petroleum ether (b.p. 40–60°C; Fisher Scientific, Loughboroough, Leicestershire, UK) was used frequently as a solvent during chemical synthesis (see below). Other chemicals were synthesized. Nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$ on a JEOL EX270 machine at 270 MHz for ^{1}H and 67.8 MHz for ^{13}C. Infrared (IR) spectra were recorded as thin films with a Perkin Elmer 298 grating spectrophotometer (Perkin Elmer, Beaconsfield, Buckinghamshire, UK).

2-Acetoxyundecane Acetylation of 2-undecanol with acetic anhydride in pyridine gave 2-acetoxyundecane (96%; b.p. 90°C/0.04 mmHg). Racemic 2-acetoxy-5-undecanone (I) was synthesized (Fig. 1) from a series of four key intermediates listed as follows.

1-Undecen-5-one Lithium hydroxide (7.2 g, 0.3 mol) was added to a stirred solution of 4-pentenoic acid (30 g, 0.3 mol; Sigma-Aldrich) in methanol (300 ml). There was a slight exotherm, and the solid dissolved after 30 min. After stirring for 2 h, the solvent was removed on a rotary evaporator and then a high vacuum pump to give the lithium salt as a white solid (32.0 g, 0.3 mol). Hexyl lithium was prepared from hexyl bromide (82.5 g, 0.5 mol) and lithium (7.7 g, 1.1 mol) in dry ether (300 ml) under

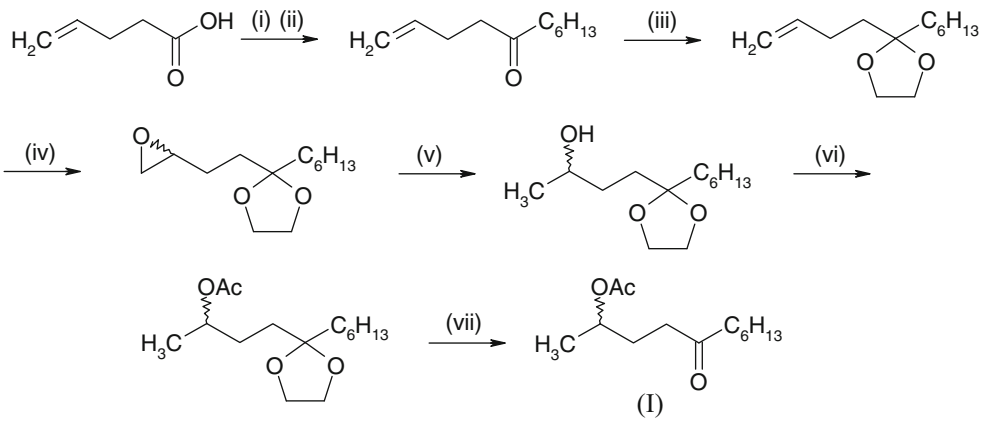

Fig. 1 Synthesis of 2-acetoxy-5-undecanone; reagents: *(i)* lithium hydroxide/methanol (100%), *(ii)* hexyl lithium/ether (83%), *(iii)* ethane diol/*p*TSA/toluene; *(iv)* 3-chloroperbenzoic acid/CH_2Cl_2; *(v)* lithium aluminum hydride/ether; *(vi)* acetic anhydride/pyridine, *(vii)* acetone/*p*TSA (76%)

nitrogen. Double titration gave a concentration of 1.1 M. The hexyl lithium (300 ml, 0.330 mol) was added dropwise to a mechanically stirred suspension of the lithium 4-pentenoate in dry ether (200 ml) under nitrogen and cooled in an ice bath. After stirring at room temperature for 48 h, the contents were poured onto ice and saturated ammonium chloride solution. The aqueous layer was extracted 2× with ether, and the extracts were washed with saturated ammonium chloride solution, dried with magnesium sulfate, and filtered through a pad of silica gel (20 g). The solvent was evaporated under reduced pressure to give the crude product (62 g), which was distilled to give a main fraction of 1-undecen-5-one (42 g, 0.25 mol, 83%) boiling at 116°C/20 mmHg. IR (film): ν 1,710 cm^{-1}; MS: *m/z* (%) 169 (10), 113 (65), 98 (18), 85(30), 83 (35), 70 (10), 55 (98), 43 (100), 41 (60).

1,2-Epoxy-5-(1,2-ethylenedioxy)undecane Ethylene glycol (37.2 g, 0.6 mol) was added to a solution of 1-undecen-5-one (42 g, 0.25 mol) in toluene (350 ml) and *p*-toluenesulfonic acid (100 mg) added as catalyst. The mixture was refluxed under a Dean and Stark head for 8 h, cooled, washed 2× with saturated aqueous sodium bicarbonate solution, and the solvent was removed on a rotary evaporator to give the ketal (65 g). MS: *m/z* (%) 157 (77), 127 (100), 99 (7), 83 (6), 55 (30), 43 (25). This was dissolved in dry dichloromethane (300 ml), and 3-chloroperbenzoic cid (77%, 57.5 g, 0.25 mol) was added portionwise to the stirred solution with ice cooling. After stirring at room temperature overnight, most of the dichloromethane was removed on a rotary evaporator, and the residue was taken up in 20% diethyl ether in petroleum ether (b.p. 40–60°C; 250 ml). After washing 2× with 2 N aqueous potassium hydroxide and drying over anhydrous potassium carbonate, the solution was filtered through silica gel (10 g), and solvents were removed on a rotary evaporator to give the epoxyketal product (58 g, 0.25 mol). ^{1}H NMR: δ 0.87 (t, *J*=6.7 Hz, 3H), 1.2–1.4 (m, 8H), 1.55–1.7 (m, 4H), 1.7–1.85 (m, 2H), 2.48 (dd, *J*=5.1, 2.6 Hz, 1H), 2.75 (dd, *J*=5.1, 4.0 Hz, 1H), 2.93 (m, 1H), 3.93 (s, 4H); ^{13}C NMR: δ 14.09, 22.60, 23.82, 26.95, 29.58, 31.81, 33.04, 37.33, 47.20, 52.26, 64.99, 111.30; MS: *m/z* (%) 157 (100), 143 (88), 125 (5), 113 (18), 99 (72), 86 (10), 69 (8), 55 (35), 43 (55), 41 (50).

2-Hydroxy-5-(1,2-ethylenedioxy)undecane 1,2-Epoxy-5-(1,2-ethylenedioxy)-undecane (58 g 0.25 mole) was dissolved in diethyl ether (400 ml) and stirred in an ice bath under nitrogen, while lithium aluminum hydride (6 g, 0.15 mol) was added portionwise. After stirring for an additional 2 h at room temperature, the reaction was carefully quenched with water (6 ml), 2 N aqueous potassium hydroxide (6 ml), and water (18 ml). Solids were filtered off, the solution dried with magnesium sulfate and filtered, and solvents were removed on a rotary evaporator to give the ketal alcohol (55.6 g). IR (film): ν 3,400 cm^{-1}; ^{1}H NMR: δ 0.88 (t, *J*=6.7 Hz), 1.19 (d, *J*=6.2 Hz, 3H), 1.25–1.4 (m, 6H), 1.45–1.65 (m, 4H), 1.65–1.85 (m, 4H), 2.14 (br d, *J*=3.7 Hz, 1H). 3.79 (br s, 1H), 3.95 (s, 4H); ^{13}C NMR: δ 14.09, 22.60, 23.53, 23.85, 29.58, 31.83, 33.23, 33.28, 37.11, 64.86, 64.89, 68.13, 111.80; MS: *m/z* (%) 168 (10), 157 (100), 145 (85), 127 (29), 111 (46), 98 (23), 83 (35), 69 (10), 55 (69), 43 (54), 41 (23).

2-Acetoxy-5-(1,2-ethylenedioxy)undecane The crude product (55.6 g) was reacted with pyridine (40 ml) and acetic anhydride (40 ml) overnight at room temperature. The mixture was dissolved in 20% diethyl ether in petroleum ether (250 ml), washed 2× with saturated sodium chloride solution and finally with a little dilute aqueous hydrochloric acid until washings were acidic. The solution was dried with magnesium sulfate, and solvents were removed on a rotary evaporator to give the acetoxyketal (66.5 g). IR (film): ν 1,725 cm^{-1}; ^{1}H NMR: δ 0.88 (t, *J*=6.7 Hz, 3H), 1.21 (d, *J*=6.2 Hz, 3H), 1.25–1.4 (m, 8H), 1.5–1.7 (m, 6H), 2.03 (s, 3H), 3.92 (s, 4H), 4.89 (hex, *J*=6.6, 1H); ^{13}C NMR: δ 14.09, 20.02, 21.36, 22.60, 23.78, 29.57, 30.15, 31.83, 32.81, 37.27, 64.63, 64.95, 70.98, 111.44, 170.71; MS: *m/z* (%) 212 (2),

167 (55), 157 (100), 127 (54), 113 (5), 99 (10), 87 (25), 83 (20), 69 (4), 55 (20), 43 (46), 41 (10).

2-Acetoxy-5-undecanone (I) The acetoxyketal was dissolved in acetone (300 ml) and stirred with *p*-toluenesulfonic acid (100 mg) for 24 h. Saturated sodium bicarbonate solution (20 ml) was added, and most of the acetone was removed on a rotary evaporator. The residue was taken up in 10% diethyl ether in petroleum ether (250 ml), washed 2× with saturated sodium chloride solution, dried over magnesium sulfate, filtered through silica gel (10 g), and solvents were removed on a rotary evaporator. The residue was distilled under reduced pressure to give a main fraction of the acetoxyketone (I) (43.0 g, 0.19 mol, 76% from 1-undecen-5-one, 63% overall) boiling at 86–90°C/0.03 mmHg). IR (film): ν 1,730, 1,710 cm^{-1}; ^{1}H NMR: δ 0.88 (t, *J*=6.5 Hz, 3H), 1.22 (d, *J*=6.2 Hz, 3H), 1.25–1.35 (m, 6H), 1.5–1.6 (m, 2H), 1.75–1.9 (m, 2H), 2.02 (s, 3H), 2.39 (t, *J*=7.6 Hz, 2H), 2.43 (t, *J*=7.6 Hz, 2H), 4.89 (hex, *J*=6.2 Hz, 1H); ^{13}C NMR: δ 14.03, 20.06, 21.28, 22.49, 23.82, 28.91, 29.73, 31.60, 38.55, 42.92, 70.30, 170.68, 210.19; MS: *m/z* (%) 229 (1), 185 (7), 168 (8), 128 (5), 113 (20), 101 (40), 98 (28), 85 (12), 83 (15), 71 (3), 55 (16), 43 (100), 41 (26).

Enantiomers of 2-Acetoxy-5-undecanone (I) The pure enantiomers were produced by separating the racemic compound by high-performance liquid chromatography (HPLC) on a column with an enantioselective stationary phase; by hydrolytic kinetic resolution of the epoxide intermediate in the synthesis (Tokunaga et al. 1997) (Fig. 2); and by enzymatic resolution.

Separation of Enantiomers of 2-Acetoxy-5-undecanone (I) by High-Performance Liquid Chromatography The racemic material (1 mg in 10 μl of hexane) was separated on a Chiralpak AD column (10×250 mm; Diacel Chemical Industry, Tokyo, Japan) eluted with 2% 2-propanol in hexane (1 ml/min) and monitored with a refractive index detector. The enantiomers eluted at 26.29 and 30.79 min. The separated enantiomers had enantiomeric excess (e.e.) of 96.1% and 98.0%, respectively, by GC analysis (Chirasil-DEX CB column).

Synthesis of Enantiomers of 2-Acetoxy-5-undecanone (*R,R*)-*N,N′*-bis(3,5-di-*tert*-butylsalicylidene)-1,2-cyclohexane-diaminocobalt (20 mg; Jacobsen catalyst; Sigma-Aldrich, Gillingham, Dorset, UK) was dissolved in toluene (200 μl) and acetic acid (35 μl) and stirred in air at room temperature for 1 h. Solvent was removed with a vacuum pump and the residue was taken up in tetrahydrofuran (2 ml). To this was added 1,2-epoxy-5-(1,2-ethylenedioxy)-undecane (0.9 g, 3.9 mM) and water (58 mg, 3.2 mM), and the mixture was stirred at room temperature. The progress of the reaction was monitored by GC (Chirasil-DEX CB column). Neither the enantiomers of the epoxyketal nor those of the 2-hydroxy-5-(1,2-ethylenedioxy)undecane were separated on this column, but those of the corresponding acetate were well separated. Accordingly, aliquots of the reaction mixture had to be worked up, reduced with lithium aluminum hydride, and then acetylated for analysis. After 72 h, the remaining epoxide was essentially one enantiomer, and the reaction was quenched with diethyl ether (10 ml) dried over magnesium sulfate, and solvents were removed on a rotary evaporator. The residue (0.85 g) was chromatographed on silica gel (30 g), and the *R*-epoxide (0.35 g) was eluted with 20% diethyl ether in petroleum ether. The corresponding diol (0.3 g) was eluted with 50% diethyl ether in petroleum ether. The epoxide was reduced with lithium aluminum hydride (38 mg, 1 mM) in diethylether (5 ml) to the alcohol (0.3 g), which was acetylated with acetic anhydride (0.2 ml) in pyridine (0.2 ml). The acetate (0.4 g) was reacted for 24 h with acetone (10 ml) and catalytic *p*-toluenesulfonic acid, and the product was Kugelrohr distilled (110°C/0.04 mmHg) to give the (*S*)-2-acetoxy-5-undecanone (0.19 g, 44%) with e.e. 93.6%. The *R*-enantiomer was prepared similarly by using the *S,S* Jacobsen catalyst and also had an e.e. of 93.6%.

Enzymatic Resolution of 2-Acetoxy-5-undecanone (I) Lipase from *Candida antarctica* on acrylic resin (25 mg; Sigma-Aldrich) was added to racemic 2-acetoxy-5-undecanone (0.456 g, 2 mM) in phosphate buffer (5 ml, pH 7), and the mixture was stirred vigorously at room temperature. Hydrolysis of the *R*-enantiomer was followed by GC analysis (Chirasil-DEX CB column) and essentially stopped after 3 h. The reaction was then extracted 3× with 20% diethyl ether in petroleum ether, the extracts were dried over magnesium sulfate, and solvents were removed on a rotary evaporator. The residue (0.4 g) was separated by flash chromatography on silica gel (20 g) eluted with 10%

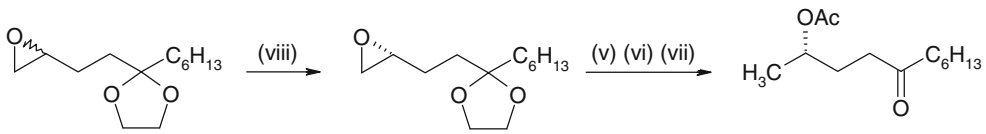

Fig. 2 Synthesis of enantiomers of 2-acetoxy-5-undecanone (*S*-enantiomer shown); reagents *(viii)* (*R*,*R*)-*N*,*N*′-bis(3,5-di-tert-butylsalicylidene)-1,2-cyclohexane-diaminocobalt (Jacobsen's reagent), acetic acid, THF, water, and chromatography, *(v)* lithium aluminum hydride/ether, *(vi)* acetic anhydride/pyridine, *(vii)* acetone/*p*TSA

and 20% diethyl ether in petroleum ether. The acetate fraction was Kugelrohr distilled (110°C/0.03 mmHg) to give the *S*-enantiomer (0.19 g, 83%) with e.e. 95.2%. The alcohol fractions were acetylated with acetic anhydride (0.25 ml) and pyridine (0.25 ml) at room temperature overnight. Aqueous workup and Kugelrohr distillation gave the *R*-acetate (0.17 g, 75%) with e.e. 96.8%.

Enzymatic Resolution of 2-Acetoxyundecane A similar treatment of racemic 2-acetoxyundecane with lipase from *C. antarctica* for 7 h gave (*S*)-2-acetoxyundecane (b.p. 70°C/0.04 mmHg; 86% yield, 97.0% e.e.) and (*R*)-2-undecanol. The latter was acetylated with acetic anhydride and pyridine to give (*R*)-2-acetoxyundecane (b.p. 70°C/0.04 mmHg; 79% yield, 99.8% e.e.).

Field Tests Field trapping tests were carried out in commercial raspberry plantations in Kent, UK. Traps were white delta traps (20×20 cm, Agrisense, Treforest, Pontypridd, UK) suspended 30–50 cm above ground level and at least 10 m apart. Pheromone dispensers were red rubber septa (20×10 mm o.d., International Pheromone Systems, Wirral, UK) or closed polyethylene vials (26×8×1.5 mm thick, Just Plastics, London, UK) impregnated with the pheromone applied in solution in petroleum ether (0.1 ml). The formulation contained 2,6-di-*tert*-butyl-4-methylphenol (BHT, 99%; Sigma-Aldrich) as an antioxidant at 20% relative to the major component.

In a first experiment, traps were baited with racemic 2-acetoxy-5-undecanone (100 μg) alone or with 2-undecanone, racemic 2-undecanol, and racemic 2-acetoxyundecane (30 μg each) in the same dispenser, released from either rubber septa or polyethylene vials. Four treatments and an unbaited control were arranged in a randomized complete block design with ten replicates (blocks). Lures were changed at 1-week intervals, and weekly counts of *R. theobaldi* males caught in each trap were made from 11 May until 7 June 2005. The total count data across 5 weeks were analyzed by using a $\log_{10}(x+1)$ transformation, the residual plot from which indicated improved and adequate variance homogeneity compared to the analysis of the untransformed data (ANOVA; Genstat for Windows version 10, VSN International, Hemel Hempstead, UK). The treatment structure used in the analysis was a control plus two dispensers × two blends factorial, and variance ratio tests were carried out on the resultant single *df* contrasts.

A second experiment was conducted in September 2005 to test the attractiveness of the individual enantiomers of 2-acetoxy-5-undecanone, separated by HPLC, in comparison with racemic 2-acetoxy-5-undecanone and blends of the latter with 2-undecanone, racemic 2-undecanol, and racemic 2-acetoxyundecane. Dispensers were rubber septa loaded with 0.01 or 0.1 μg of the major component. Eight treatments and an unbaited control were arranged in a randomized complete block design with six replicates (blocks). Weekly counts were made of the numbers of *R. theobaldi* males caught in each trap over a 3-week period until the flight period ended. The total counts were analyzed after $\log_{10}(x+1)$ transformation, which again produced adequate residual plots to indicate that the assumption of variance homogeneity was achieved. Two types of ANOVA were done. The first was an ANOVA with no partitioning of treatment factors followed by a Duncan's multiple range test to separate means (α=0.05). In the second, treatment factors for control and treated, racemic and enantiomer, loading, presence or absence of minor component were included in the analysis, which subdivided the 8 *df* for treatments into single *df*s to assess the various effects explicitly. Following this analysis, means were separated by a least significant difference test (α=0.05).

Measurement of Release Rates from Dispensers Two of each rubber septa and polyethylene vial dispensers loaded with the four-component blend containing 2-acetoxy-5-undecanone (100 μg), and 2-undecanone, 2-acetoxyundecane, and 2-undecanol (30 μg each) were maintained in a laboratory wind tunnel under constant conditions of 27°C and 8 km/h air speed. At intervals, volatiles released from the individual dispensers during 2–4 h were collected on Porapak Q resin, as described above for collection of volatiles from live insects. The collections were analyzed by GC-FID (Wax10 column) with dodecyl acetate (5 μg) as internal standard.

Results

GC-MS Analyses Analyses of collections of volatiles from male and female *R. theobaldi* showed four components in collections from females that were not present in collections from males (Fig. 3). These were a major component (I) and three earlier-eluting minor components (II, III, IV), each present at approximately 30% of the major component. Retention indices for these compounds were calculated relative to the retention times of normal hydrocarbons (Table 1).

The three earlier-eluting minor components were identified as 2-undecanone (II), 2-acetoxyundecane (III), and 2-undecanol (IV) from the spectral libraries, and by subsequent comparison of retention times and mass spectra with authentic standards. 2-Undecanone and 2-undecanol are commercially available, and 2-acetoxyundecane was synthesized by acetylation of 2-undecanol. Further examination of the chromatograms showed that the corresponding 9-carbon compounds, 2-nonanone, 2-acetoxynonane, and 2-nonanol were present at 1–2% of the major component in the collections from females, but not in collections from males (data not shown).

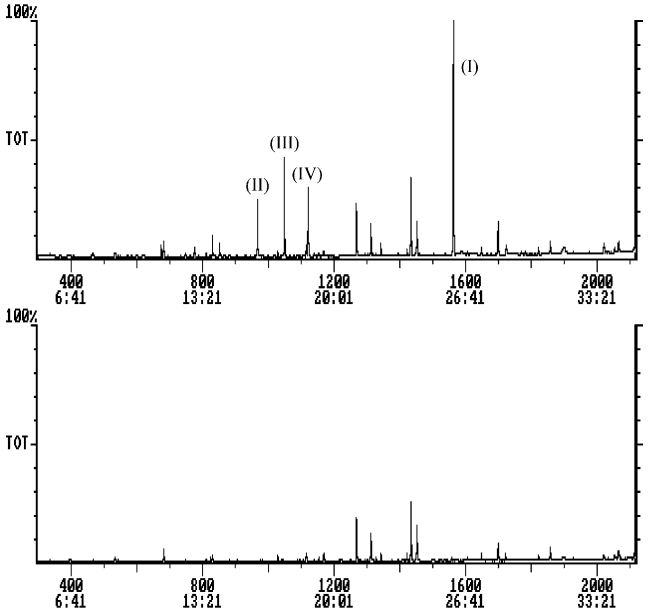

Fig. 3 GC-MS analyses on Wax10 GC column of volatiles collected from female (*upper*) and male (*lower*) *R. theobaldi* showing female-specific components

Comparison of peak areas with those of standard acetates indicated that the major component (I) was present at approximately 1.5 μg in a collection from a total of 200 females over a 1-week period. This is more than 30 times the amount of pheromone obtained from females of the apple leaf curling midge, *D. mali*, under similar conditions (Cross and Hall 2005; Hall et al. 2005).

GC-EAG Analyses In GC-EAG analyses of the collections of volatiles from female midges with a male midge EAG preparation, an EAG response always was observed to the major female-specific component. In some cases, there were also responses to 2-acetoxyundecane (III) and 2-undecanol (IV) (Fig. 4).

Chemical Structure of the Major Pheromone Component The difference in retention indices for the major female-specific component on the polar and nonpolar GC columns (Table 1) indicated that this was much more polar than any of the minor components and probably had the dioxygenated functionality characteristic of several midge pheromones identified to date. The difference in retention indices on polar and nonpolar columns for this component was intermediate to those of a diacetate structure such as 2,12-diacetoxytridecane, a component of the pheromone of *C. pisi* (Hillbur et al. 1999), and an unsaturated acetoxyketone structure such as (*Z*)-13-acetoxy-8-heptadecen-2-one, a component of the pheromone of *D. mali* (Cross and Hall 2005; Hall et al. 2005; Table 1).

The EI and CI mass spectra (Fig. 5) did not show a clear molecular ion, although the highest ion at *m/z* 229 in the ion trap spectrum suggested a molecular weight of 228 corresponding to an 11-carbon, saturated acetoxyketone. The ion at *m/z* 169 could be due to the loss of acetic acid from an acetate, as further evidenced by the ions at *m/z* 43 and 61. Hydrolysis of this component with potassium carbonate in methanol gave a product with longer GC retention time (RI 2117 on the polar column) giving a very broad peak.

Further analysis of the EI mass spectrum indicated this component to be 2-acetoxy-5-undecanone, the structure of which could explain all the main ions in the mass spectrum (Fig. 6). Hydrolysis of this would give the corresponding keto-alcohol, which could exist in cyclic or acyclic forms and might be expected to give a broad peak on GC analysis.

This compound was synthesized in seven steps (Fig. 1) and found to have identical GC retention times and mass spectra to those of the natural, female-specific component.

Stereochemistry of Pheromone Components The enantiomers of the chiral pheromone components were separated by enantioselective GC. Separations of the enantiomers of 2-acetoxyundecane and 2-acetoxy-5-undecanone were very good, but the enantiomers of 2-undecanol were only poorly separated (Fig. 7).

In analyses of the collections of volatiles from female *R. theobaldi* (Fig. 7), only a peak at the retention time of the first eluting enantiomer of the major pheromone component was present; the later-eluting enantiomer could not be detected (<0.01%). Similarly, only the first-eluting enantiomer of 2-acetoxyundecane was present; a smaller peak had a similar but significantly different retention time, but the possibility of a small amount (≤5% of the first-eluting enantiomer) of the second enantiomer being present cannot be excluded. Enantiomers of 2-undecanol were poorly

Table 1 GC retention data from GC-MS analyses of female-specific components of *R. theobaldi* and some synthetic pheromone components of other midges

Female-specific compound	Compound	Retention index[a]		
		Wax10	SPB1	Δ[b]
(II)	2-Undecanone	1600	1275	325
(III)	2-Acetoxyundecane	1665	1422	243
(IV)	2-Undecanol	1722	1290	432
(I)	2-Acetoxy-5-undecanone	2091	1549	542
	2,12-Diacetoxytridecane	2448	1955	493
	(*Z*)-13-Acetoxy-8-heptadecen-2-one	2761	2110	651

[a] Retention index (RI) relative to retention times of normal hydrocarbons

[b] Difference between RIs on polar Wax 10 and nonpolar SPB1 columns

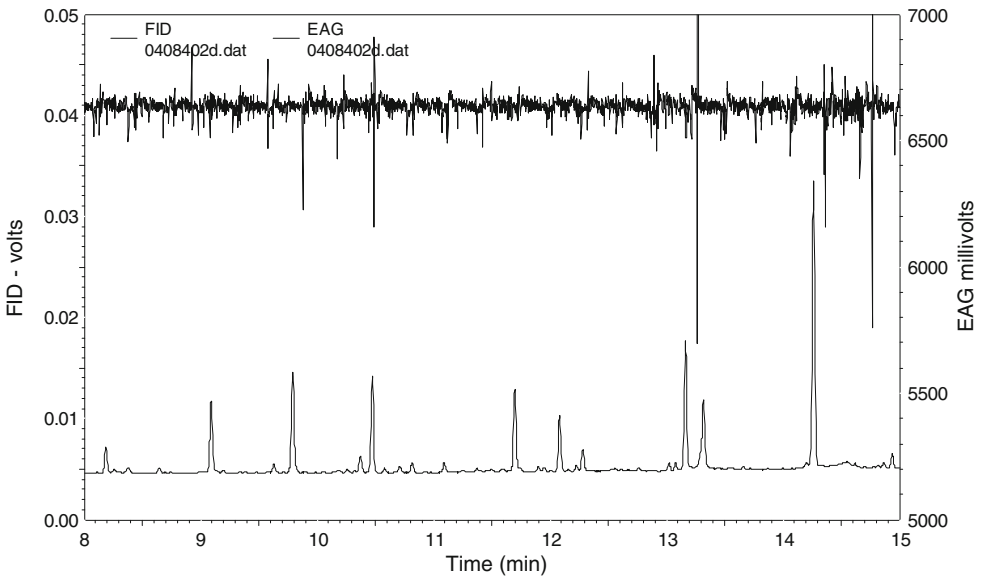

Fig. 4 GC-EAG analysis of volatiles from female *R. theobaldi* by a male *R. theobaldi* EAG preparation and Wax10 GC column (*lower trace* GC, *upper trace* EAG; EAG signals at 13.2 and 14.9 min are interference spikes)

separated, but only the first-eluting enantiomer seemed to be present in the natural pheromone collections (Fig. 7).

The enantiomers of 2-acetoxy-5-undecanone (I) were also separated by HPLC on a column with a chiral stationary phase, and milligram quantities were provided for field testing. These were analyzed by GC-FID (Chirasil-DEX CB column) and shown to have high enantiomeric purity. The enantiomer that eluted first in HPLC analyses eluted second in GC analyses.

The configurations of the enantiomers were determined by hydrolytic kinetic resolution of the epoxyketal intermediate in the synthesis of the racemic compound (Fig. 1) with the Jacobsen reagent (Tokunaga et al. 1997; Fig. 2). Reaction of the terminal epoxide with (*R*,*R*)-*N*,*N*′-bis(3,5-di-*tert*-butylsalicylidene)-1,2-cyclohexane-diaminocobalt has been reported to hydrolyze the *S*-epoxide, thus leaving the *R*-enantiomer (Tokunaga et al. 1997; Choi et al. 2004). Isolation of the *R*-epoxide followed by reduction, acetylation, and deketalization then gave (*S*)-2-acetoxy-5-undecanone (I; Fig. 2). Use of the *S,S* Jacobsen reagent gave (*R*)-2-acetoxy-5-undecanone. In GC analysis of the enantiomers, (*S*)-2-acetoxy-5-undecanone eluted first. Thus, this is the naturally occurring enantiomer.

This assignment was further confirmed by hydrolysis of the racemic 2-acetoxy-5-undecanone with lipase from *C. antarctica.* This enzyme selectively hydrolyzes the *R*-acetate (e.g., Xiao and Kitazume 1997; Gries et al. 2006). The resultant *R*-alcohol could be separated from unreacted *S*-acetate and then re-acetylated to provide a convenient preparative route to both enantiomers of the acetate in high enantiomeric purity. The unreacted *S*-acetate eluted first on the cyclodextrin GC column. The enantiomers of 2-acetoxyundecane and 2-undecanol were prepared similarly. Analysis of both by enantioselective GC showed that the *S*-enantiomers eluted first. Thus, these are the naturally occurring enantiomers.

Field Tests In the first field test, comparisons were made of catches of *R. theobaldi* males in traps baited with the racemic form of the major pheromone component, 2-acetoxy-5-undecanone, alone or combined with the three potential minor components and dispensed from rubber septa or polyethylene vials (Table 2). Over 5 weeks, total catches with all the lures were greater than those in the unbaited control ($F_{1,36}$=299.75; P<0.001), but there were no significant differences among catches with the different

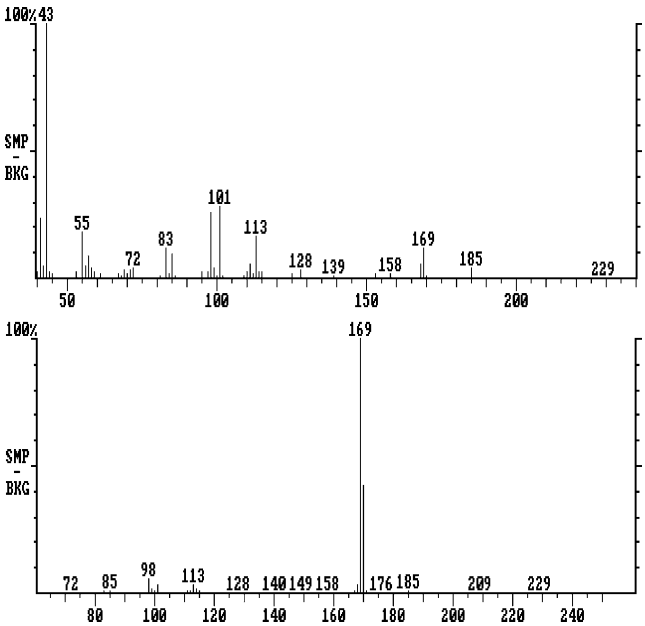

Fig. 5 Mass spectra of the major component of the sex pheromone of *R. theobaldi* in EI (*upper*) and CI (*lower*) modes

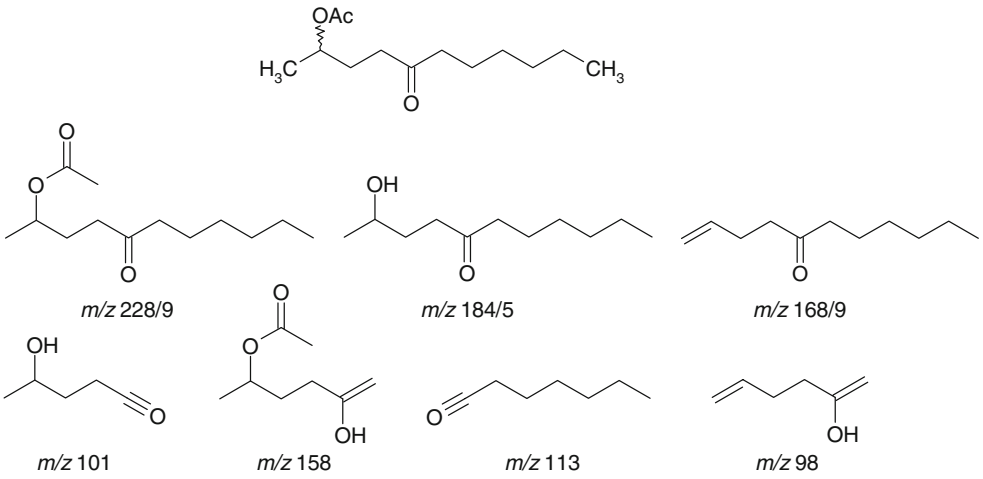

Fig. 6 Proposed structure for 2-acetoxy-5-undecanone, the major female-specific sex pheromone component in *R. theobaldi* and assignment of fragments in the mass spectrum

blends ($F_{1,36}$=3.35; P=0.076) or dispensers ($F_{1,36}$=0.017; P=0.686). The interaction between the two treatment factors also was not statistically significant ($F_{1,36}$=1.66; P=0.205).

In the second field test, total numbers of male *R. theobaldi* caught were rather small, but the treatment effect was clear (Table 3). Analysis of variance of the transformed data without inclusion of treatment factors after $\log_{10}(x+1)$ transformation gave a highly significant treatment effect ($F_{8,40}$=18.18; P<0.001). Traps baited with (*R*)-2-acetoxy-5-undecanone did not catch more *R. theobaldi* than the unbaited control at either loading (P>0.05, Duncan's multiple range test). The highest loading (100 ng) of the *S*-enantiomer or the racemate alone or with minor components added caught the largest and similar numbers of *R. theobaldi* (P>0.05). These attractive treatments at the lower load (10 ng) caught lower numbers of males, but still caught significantly more than the unbaited control traps (P<0.05). Traps baited with 10 ng of racemic 2-acetoxy-5-undecanone caught more (P<0.05) male *R. theobaldi* than those baited with 10 ng of the *S*-enantiomer, which may reflect the variability expected with the relatively low catches.

The second ANOVA of these data, which focused on treatment factors, showed that there was a strong overall dose effect for the racemic lures ($F_{1,40}$=13.31; P<0.001), with high dose > low dose > control and all differences significant (P<0.01, least significant difference test). There was no statistically significant evidence of an effect of adding the minor components ($F_{1,40}$=2.04; P=0.161) nor of any interaction with dose ($F_{1,40}$=1.08; P=0.304). There was a strong overall difference between enantiomers ($F_{1,40}$= 38.76; P<0.001) and a dose effect ($F_{1,40}$=10.76; P=0.002), but also an interaction ($F_{1,40}$=13.11; P<0.001), presumably because there was no dose effect for the inactive *R*-enantiomer, but a strong one for the active *S*-enantiomer where high dose > low dose > control (P<0.05).

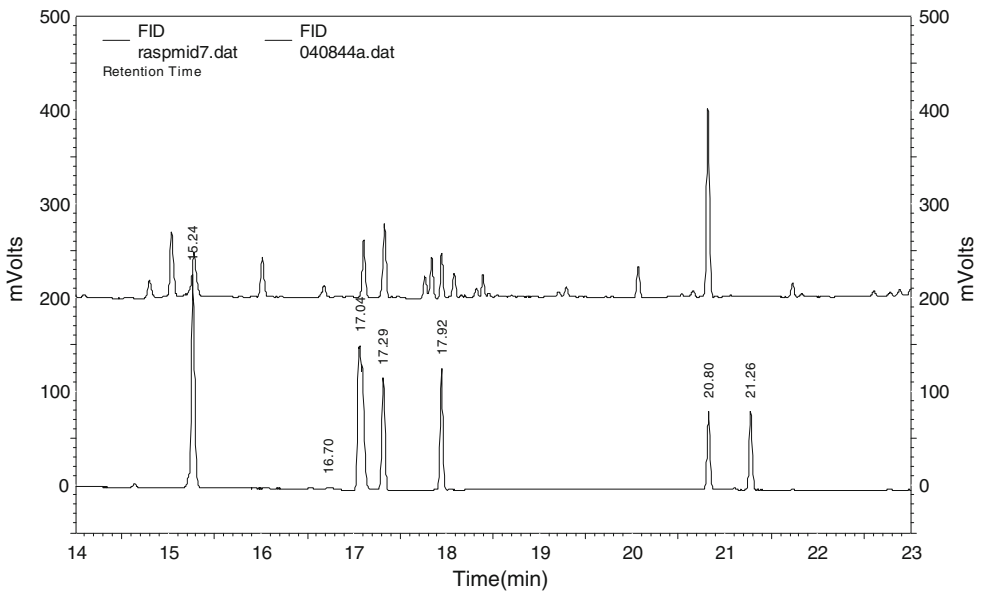

Fig. 7 GC analyses on cyclodextrin column of synthetic standards (*lower*) and volatiles from female *R. theobaldi* (*upper*) [2-undecanone *(II)* at 15.24 min; 2-undecanol *(IV)* at 17.04 and 17.06 min; 2-acetoxyundecane *(III)* at 17.29 and 17.92 min; 2-acetoxy-5-undecanone *(I)* at 20.80 and 21.26 min]

Table 2 Mean total numbers of male *R. theobaldi* caught between 11 May and 14 June 2005, in commercial raspberry plantations, Kent, UK

Component[a] (μg)				Dispenser[c]	Mean total catch[b]	
(I)	(II)	(III)	(IV)		*x*	log(*x*+1)
100	–	–	–	RS	235.2	2.012
100	–	–	–	PV	264.8	2.096
100	30	30	30	RS	356.2	2.310
100	30	30	30	PV	265.4	2.148
Unbaited					1.5	0.290
SED (36 *df*)						0.1352

[a] Components: *(I)* 2-acetoxy-5-undecanone, *(II)* 2-undecanone, *(III)* 2-acetoxyundecane, *(IV)* 2-undecanol
[b] *x*=mean total trap catch (*N*=10 replicates); $\log_{10}(x+1)$=transformed mean total trap catch (*N*=10 replicates); *SED* standard error of the difference between means
[c] Pheromone dispensers: *RS* rubber septum, *PV* polyethylene vial

Measurement of Release Rates from Dispensers Laboratory wind tunnel release rate measurements showed that the rubber septa dispensers had a roughly exponential decline in release rate of 2-acetoxy-5-undecanone (Fig. 8). The three minor components were released at approximately 30% of the rate of the major component. Release of the major component from the polyethylene vials increased during the first 10 days, remained relatively constant for the next 15 days, and then declined. Release of the minor components declined relatively evenly throughout. Apart from changes in overall release rate, the ratio of major to minor components varied dramatically from approximately 1:1 to 3:1, and back to 1:1 during the experiment. The release rate from both dispensers had declined to 10% of the maximum after 33 days at 27°C. Hence, lures should remain active for at least 1 month in the field.

Discussion

In this study, virgin females of the raspberry cane midge, *R. theobaldi*, were shown to produce (*S*)-2-acetoxy-5-undecanone accompanied by smaller amounts of 2-undecanone, (*S*)-2-acetoxyundecane, and (*S*)-2-undecanol. The major component elicited an EAG response from conspecific male midges and was highly attractive to male midges in field trapping tests. Thus, we propose that (*S*)-2-acetoxy-5-undecanone is the major component of the female sex pheromone of this species.

The structure of 2-acetoxy-5-undecanone is related to those of components of the pheromones of other midge species, having an odd number of carbon atoms in the chain and two oxygenated functionalities, one at the 2-position. It is the second example of a midge pheromone component with a ketone functional group, the only other such

Table 3 Mean total numbers of male *R. theobaldi* caught between 7 September and 4 October 2005, in commercial raspberry plantations, Kent, UK

Component[a] (ng)				Mean total catch[b]	
(I)	(II)	(III)	(IV)	*x*	Log(*x*+1)[c]
100 *RS*	–	–	–	22.2	1.302 ab
10 *RS*	–	–	–	9.8	0.983 bc
100 *RS*	30	30	30	20.2	1.255 ab
10 *RS*	3	3	3	5.8	0.680 cd
100 *R*	–	–	–	0.7	0.159 ef
10 *R*	–	–	–	1.0	0.201 ef
100 *S*	–	–	–	24.7	1.366 a
10 *S*	–	–	–	3.3	0.520 de
Unbaited	–	–	–	0.2	0.050 f
SED (40 *df*)					0.1734

[a] Components: (*I*) 2-acetoxy-5-undecanone (*R*- or *S*-enantiomer or racemic *RS*), (*II*) 2-undecanone, (*III*) racemic 2-acetoxyundecane, (*IV*) racemic 2-undecanol
[b] *x*=mean total trap catch (*N*=6 replicates), $\log_{10}(x+1)$=transformed mean total trap catch (*N*=6 replicates)
[c] Means followed by the same letter are not significantly different by Duncan's multiple range test at 5% level; *SED* standard error of the difference between means

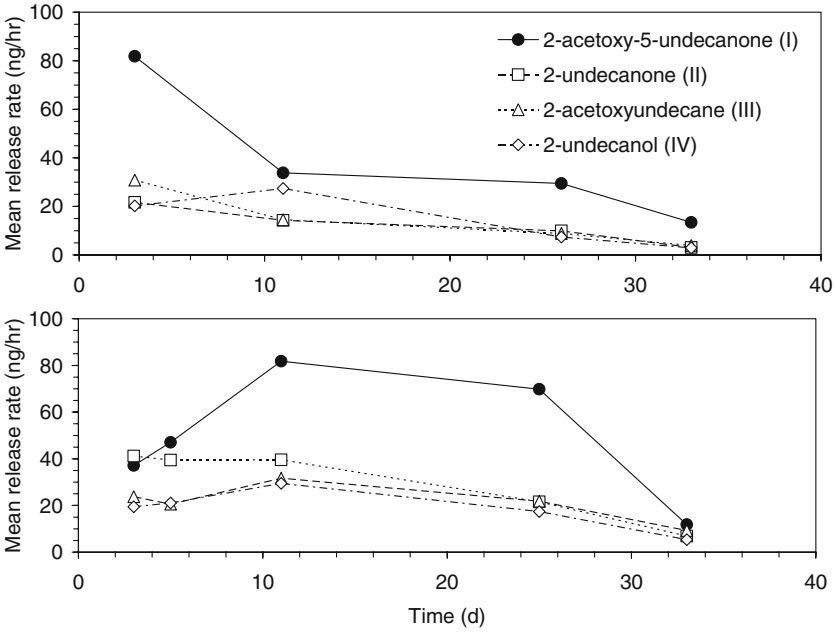

Fig. 8 Release rates of synthetic pheromone components from rubber septa (upper) and polyethylene vials (lower) measured at 27°C by collection of volatiles on Porapak Q

compound being (*Z*)-13-acetoxy-8-heptadecen-2-one, the pheromone of the apple leaf curling midge, *D. mali* (Cross and Hall 2005, 2009; Hall et al. 2005; Suckling et al. 2007; Cross et al. 2009b).

Female *R. theobaldi* produce (*S*)-2-acetoxy-5-undecanone, and this enantiomer is attractive to males. The *R*-enantiomer is not attractive, but the racemic mixture is just as attractive as the *S*-enantiomer. This is the same situation as in other midge pheromones with a single chiral center, i.e., (*S*)-(*Z*,*Z*)-2-acetoxy-4,7-tridecadiene in the Douglas-fir cone gall midge, *C. oregonensis* (Gries et al. 2002); (*R*)-2-acetoxy-8-heptadecene in the locust bean midge, *Dasineura gleditchiae* (Molnár et al. unpublished data); and (*S*)-2-butyroxy-8-heptadecene in the Chinese chrysanthemum midge, *Rhopalomyia* spp. (Liu et al. unpublished data). With (*Z*)-13-acetoxy-8-heptadecen-2-one in *D. mali*, both the racemate and one of the enantiomers are equally attractive to male midges, although work is still in progress to confirm the configuration of the attractive enantiomer (Cross and Hall 2005; Hall et al. 2005). This contrasts with the situation with most midge pheromones with two chiral centers where typically one diastereomer is attractive, and one or more of the others strongly inhibits attraction. Thus, in the pea midge, *C. pisi*, a blend containing (*S,S*)-2,11-diacetoxytridecane and (*S,S*)-2,12-diacetoxytridecane is highly attractive to male midges, but addition of small amounts of the 2*S*,11*R*- or 2*R*,11*S*-isomers of the former component completely eliminates attraction (Hillbur et al. 2001).

2-Undecanone, (*S*)-2-acetoxyundecane, and (*S*)-2-undecanol are also produced by female *R. theobaldi*, and at least the latter two components elicited EAG responses from a male midge. However, addition of these as the racemic forms to racemic 2-acetoxy-5-undecanone did not have any significant effect on attractiveness. This contrasts with results for *C. pisi* where addition of racemic 2-acetoxytridecane to the blend of (*S,S*)-2,11-diacetoxytridecane and (*S,S*)-2,12-diacetoxytridecane is essential for attraction of male midges (Hillbur et al. 2000). Further work will be carried out to determine whether addition of the *S*-enantiomers of the potential minor components to the (*S*)-2-acetoxy-5-undecanone, as in the naturally produced blend, has any effect on the attractiveness of the latter component.

The pheromone of *R. theobaldi* was produced in relatively large quantities compared with that obtained from other midge species. This may have been due in part to the use of air entrainment to collect pheromone rather than gland extraction, which has been used in most other midge pheromone identifications (e.g., Hillbur et al. 1999). Air entrainment allows large numbers of midges to be processed easily, and gives a collection free of involatile material that can be concentrated for GC analysis. Thus, collection of pheromone from a total of 200 female *R. theobaldi* gave approximately 1.5 μg of the major component. However, this was more than 30 times the amount of pheromone collected from another midge species, *D. mali*, under identical conditions, so the larger amounts were not entirely due to the method of collection.

Racemic 2-acetoxy-5-undecanone was synthesized on multigram scale in seven steps and 63% overall yield from commercially available 4-pentenoic acid (Fig. 1). The basic ketone structure was set up by using reaction of an organolithium reagent with a carboxylic acid (Jorgenson 1970). The utility of this reaction has been demonstrated previously (Hall et al. 2006). As in the latter case, use of the preformed lithium salt of the carboxylic acid is more

economical in organolithium reagent and, in this case, produces a higher yield of cleaner product.

The enantiomers of 2-acetoxy-5-undecanone can be separated not only by enantioselective GC but also by HPLC. A normal-phase Chiralpak AD column (Qin et al. 1997; Gries et al. 2000) was used, and milligram quantities were produced for the field tests. The configurations of the enantiomers were determined by hydrolytic kinetic resolution of the terminal epoxide intermediate in the synthesis of the racemic material by using Jacobsen's reagent, *N*,*N*′-bis(3,5-di-*tert*-butylsalicylidene)-1,2-cyclohexane-diaminocobalt (Tokunaga et al. 1997). The *R*,*R*-reagent hydrolyzes the *S*-epoxide leaving the *R*-epoxide, which is isolated and converted to the (*S*)-2-acetoxy-5-undecanone (Fig. 2). This elutes first on the cyclodextrin GC column, as does the enantiomer produced by female *R. theobaldi*. This assignment was confirmed by hydrolysis of racemic 2-acetoxy-5-undecanone with a lipase that is known to hydrolyze selectively the *R*-enantiomer (e.g., Xiao and Kitazume 1997; Gries et al. 2006). With the ester function in the 2-position, the lipase from *C. antarctica* gives extremely high enantiomeric purity (Gries et al. 2006; Liu et al. unpublished data; Molnár et al. unpublished data). With the ester function further along the carbon chain, this lipase is less selective, and others such as Amano AK are more selective (e.g., Lacey et al. 2007; Hall et al. unpublished). Both chemical and enzymatic approaches provide convenient routes to multigram quantities of the enantiomers of 2-acetoxy-5-undecanone.

Rubber septa or polyethylene vials were used as pheromone dispensers in the first field experiment, and both proved attractive. Release rate studies on the rubber septa showed that release of 2-acetoxy-5-undecanone and the minor components, 2-undecanone, 2-acetoxyundecane, and 2-undecanol showed a "conventional" first-order decline. However, with the vials, although the minor components showed first-order release, release of the 2-acetoxy-5-undecanone took several days to reach a maximum, and then declined. This effect was also noted with (*Z*)-13-acetoxy-8-heptadecen-2-one, pheromone of *D. mali* (unpublished results) and is presumably due to the slow penetration of the more polar ketoacetate through the polyethylene. For field use, the more predictable exponential decline in release rate from the rubber septa is probably preferable. The release rate from either dispenser was still significant after 30 days in the laboratory wind tunnel at 27°C and 8 km/h airspeed. Under field conditions in the UK, where average temperatures are much lower, the life of lures is likely to be considerably longer.

Work is in progress to optimize pheromone loading, dispenser and trap design, and to investigate the use of the pheromone for control of *R. theobaldi* in commercial plantations by attract-and-kill or mating disruption. Work to correlate pheromone trap catches with field populations of the midge and plant damage was started in eight European countries in 2006 (Cross et al. 2008). A pheromone trap monitoring system and/or a nonchemical control method for control of the pest will reduce UK raspberry growers' dependence on chlorpyrifos, residues of which were found in 17% of samples of UK-produced raspberry fruit in 2003 (Anonymous 2003a, b).

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Sex Pheromone Components of the Pear Fruit Moth, *Acrobasis pyrivorella* (Matsumura)

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Abstract We analyzed the sex pheromone of the pear fruit moth, *Acrobasis pyrivorella*, by means of gas chromatography–electroantennographic detection (GC-EAD) and GC–mass spectrometry. Two EAD-active compounds were detected in the pheromone gland extract of females. They were identified as (*Z*)-9-pentadecenyl acetate (Z9-15:OAc) and pentadecyl acetate (15:OAc). The amounts per female gland (mean ± standard error) of these compounds were 12.9±2.8 and 0.8±0.1 ng, respectively. Synthetic Z9-15:OAc (300 μg) attracted conspecific males in field trapping experiments. When 15:OAc (21 μg; 7% of Z9-15:OAc quantity) was added, the number of males trapped increased significantly. Catch in traps baited with the mixture of these compounds was greater than that in traps baited with 1–3-day-old virgin females. We, therefore, conclude that Z9-15:OAc and 15:OAc are sex pheromone components of this species.

Keywords Sex pheromone · (*Z*)-9-pentadecenyl acetate · Pentadecyl acetate · Pear fruit moth · *Acrobasis pyrivorella* · *Ectomyelois pyrivorella* · Lepidoptera · Pyralidae

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Introduction

The pear fruit moth, *Acrobasis pyrivorella* (Matsumura) (Lepidoptera: Pyralidae, Phycitinae), is a serious pest of pears, *Pyrus pyrifolia* (Burman) or *Pyrus communis* L. (Rosaceae: Maloideae), indigenous to the temperate zone of eastern Asia, and is widely found in far-eastern Russia, northern China, Korea, Taiwan, and Japan (EPPO 2007). Synonyms for this insect include *Nephopterix pirivorella* Matsumura, *Nephopteryx pauperculella* (Wileman), *Acrobasis pirivorella* (Matsumura), *Ectomyelois pyrivorella* (Matsumura), *Eurhodope pirivorella* (Matsumura), *Numonia pirivora* (Gerasimov), *Numonia pyrivorella* (Matsumura), and *Rhodophaea pirivorella* (Matsumura) (Walker 2006). Currently, there is no indication that *A. pyrivorella* larvae feed on fruit trees other than pears (Walker 2006). Larvae (first–third instars) of *A. pyrivorella* feed on the developing buds or fresh flowers before boring into the core of the young fruit (Makaji 1965). Larvae may move from fruit to fruit, with a single larva capable of destroying up to two or three buds, one to three primordial flowers, and three fruit (Shutova 1977).

In Japan, the major fruit borers in pear orchards are the Oriental fruit moth *Grapholita molesta* (Busck), peach fruit moth *Carposina sasakii* Matsumura, yellow peach moth *Conogethes punctiferalis* (Guenée), and *A. pyrivorella* (Adachi 1999). The sex pheromones of *G. molesta* (Cardé

et al. 1979), *C. sasakii* (Tamaki et al. 1977), and *Con. punctiferalis* (Konno et al. 1982) have all been identified. Sex pheromones of *G. molesta* and *C. sasakii* are commercially available and used for monitoring their flight phenology (Chiba 2000; Tanaka 2000), as well as for mating disruption (Izawa 2000). However, the sex pheromone of *A. pyrivorella* has not been studied previously. In the present study, we analyzed sex pheromone gland extract of *A. pyrivorella* females by gas chromatography–electroantennographic detection (GC-EAD) and GC–mass spectrometry (GC-MS). Candidates for sex pheromone components were identified and synthesized, and the behavioral response they evoked in conspecific males was tested by field-trapping experiments.

Methods and Materials

Insects Approximately 500 fruit, damaged by *A. pyrivorella*, were collected from pear trees around Nagano Nannshin Agricultural Experiment Station (35.6°N, 137.9°E; Shimoina-gun, Nagano Pref.) from 1–8 August 2007. A total of 48 pupae were obtained from the fruit, from which 19 adult females and 23 adult males emerged. Adults were housed individually in a 200-ml plastic container with a piece of cotton soaked with water and placed in a rearing room (60%±5% relative humidity, L15:D9 h photoperiod, 23±1°C).

Extraction of Sex Pheromone Pheromone gland extract was obtained from 2-day-old virgin females in the last 2 h of the dark period, when most females had started calling behavior. The abdominal tip, which includes the sex pheromone gland, was excised and immersed in 20 µl hexane (Wako Pure Chemicals, Osaka, Japan). The tip was removed from the solvent after 30 min, and the extract was stored at −20°C before analysis. Four extracts were produced, from two, three, four, and five pheromone glands, respectively.

Gas Chromatography and Gas Chromatography–Electroantennographic Detection GC analyses were conducted on an Agilent 6890N gas chromatograph (Santa Clara, CA, USA) equipped with a split/splitless injector and a flame ionization detector (FID). The injector and detector were each kept at 220°C. An apolar HP-1 [25×0.2 mm inner diameter (ID), 0.33 µm film thickness; Agilent], a polar DB-wax (30×0.25 mm ID, 0.25 µm film thickness; J&W Scientific, Folsom, CA, USA), or a polar DB-23 (30×0.25 mm ID, 0.15 µm film thickness; J&W Scientific) column were used for analyses, at a constant flow rate of 1.0 ml min^{-1} helium carrier gas. Aliquots of samples (0.2–1.0 female equivalent) were injected into the GC system with a purge time of 1 min. In the quantitative measurement of compounds, samples were mixed with 2 ng of an internal standard (tridecyl acetate; Sigma-Aldrich, St. Louis, MO, USA) before injection. The column oven temperature was maintained at 60°C for 1 min, raised to 220°C at a rate of 7°C min^{-1}, and held for 8 min. GC-EAD (Struble and Arn 1984) was performed on a Hewlett-Packard (Palo Alto, CA, USA) 5890GC equipped with an EAD device (Taiyo, Tsukuba, Japan) and a DB-23 column. The conditions and equipment for the GC were the same as above. An antenna was excised from a virgin male (1 day old), and a few of the distal segments cut off. The two ends of the antenna were attached to the electrodes of the EAD device with a few droplets of electroconductive gel (Aquasol, Parker, Fairfield, NJ, USA).

Gas Chromatography–Mass Spectrometry GC-MS analyses were conducted on an Agilent 6890N/5973, in electron impact mode at 70 eV. The interface temperature was 230°C. The other conditions and equipment were the same as those for the GC-FID analysis.

Dimethyl Disulfide Derivatization Monounsaturated compounds in the crude sex pheromone gland extract were reacted with dimethyl disulfide (DMDS), to confirm double-bond location and geometry, according to the method of Buser et al. (1983). The crude extract in hexane (ca. five female equivalents) was treated with 100 µl DMDS (Wako) and one drop of iodine solution (60 mg/ml in ether). The reaction mixture was kept at 40°C on a heat block for 24 h. The cooled sample was diluted with 200 µl hexane. Iodine was removed by shaking with 5% aqueous $Na_2S_2O_3$ (100 µl). The organic phase was drawn off, and the aqueous phase was extracted in 200 µl hexane. The combined hexane solution was dried over anhydrous sodium sulfate, concentrated to ca. 10 µl, and subjected to GC-MS analysis.

Chemicals (*Z*)-9-pentadecenyl acetate (Z9-15:OAc) was synthesized via the Grignard cross-coupling reaction as follows: (*Z*)-9-chloro-6-nonene (Shin-Etsu Chemical, Tokyo, Japan) was reacted with magnesium metal in tetrahydrofuran (THF) to yield (*Z*)-3-nonen-1-yl magnesium chloride. This Grignard reagent was cross-coupled with 1-bromo-6-chlorohexane (Nippoh Chemicals, Tokyo, Japan) to give (*Z*)-15-chloro-6-pentadecene crude product (Yamamoto and Ishihara 1991). After evaporation of THF under reduced pressure, crude (*Z*)-15-chloro-6-pentadecene was reacted with sodium acetate (Daito Chemical, Tokyo, Japan) in glacial acetic acid to give Z9-15:OAc. The crude product was purified to 93% purity in a distillation tower. The product contained approximately 3% of the (*E*)-isomer (E9-15:OAc). These geometric isomers were purified further by high-performance liquid chromatography

(HP1050 series; Hewlett-Packard) in a silver-ion-coated column (Nucleosil 100-SA, 4.6 mm diameter × 250 mm length, 5 μm particle size; GL Science, Tokyo, Japan; Christie 1988). Hexane containing 30% ether was used as the elution solvent at a flow rate of 1 ml min^{-1}. The geometries of Z and E9-15:OAcs were confirmed by Fourier transformation infrared spectrophotometry (JIR-100; JEOL, Tokyo, Japan); E9-15:OAc had a unique absorption at 966 cm^{-1}. Both Z and E9-15:OAc samples contained less than 1% of the opposite isomer. Pentadecyl acetate (15:OAc) was prepared by acetylation of 1-pentadecanol (Tokyo Chemical Industry, Tokyo, Japan) with acetic anhydride in the presence of pyridine. The compounds were condensed under vacuum, redissolved in hexane, and prepared for use in field traps as described below.

Field Trap Experiments Delta-traps with 24×30-cm sticky boards (SE-trap; Sankei Chemical, Kagoshima, Japan), baited with synthetic chemicals or virgin females, were used for testing attractiveness to males in pear orchards around Nagano Nannshin Agricultural Experiment Station. Synthetic chemicals dissolved in ca. 100 μl hexane were impregnated onto rubber septa (gray, 8 mm diameter× 19 mm height; West, Singapore). The solvent was evaporated overnight at room temperature. Traps baited with natural virgin females contained two virgins (1–3 days old) held in a steel mesh cage (6 cm diameter × 5 cm height) with a cotton pad soaked with a 5% honey solution. Traps were hung (1.5–2.0 m above the ground) on pear trees at ca. 2–5-m intervals and checked every 2 days, after which their positions were rerandomized.

Experiments 1, 2, and 3 were conducted from 22 June to 9 July 2007 (checked nine times with one replicate), 22 to 27 August 2006 (checked three times with two replicates), and 18 to 27 August 2006 (checked five times with two replicates), respectively. Experiments 4 and 5 were conducted with three replicates from 17 June to 2 July 2008, and experiment 6 was conducted with three replicates from 5 to 20 August 2007 (each checked eight times). Data (x) were transformed as $(x+0.5)^{1/2}$ and analyzed by a two-way analysis of variance (ANOVA). Means of treatments were separated by the Tukey–Kramer honestly significant difference (HSD) test ($\alpha=0.05$) using JMP version 5.1.1 software (SAS Institute 1989–2004). Treatments that did not catch males were not included in the statistical analyses to avoid violating assumptions of ANOVA.

Results

Sex Pheromone Chemistry Two EAD-active compounds (A and B) were found in the crude extract of abdominal tips of virgin females (Fig. 1). The amounts of these compounds per female gland were 0.8±0.1 and 12.9±2.8 ng (mean ± standard error), respectively. In the GC-MS analysis of compound A, a characteristic fragment ion of aliphatic acetates (*m/z* 61; $CH_3COOH_2^+$) was observed (Fig. 2). Compound A had an ion at *m/z* 210 (M^+-60) and had a similar mass spectrum to acetates of saturated primary fatty alcohols, suggesting it was 15:OAc. Compound B also had an *m/z* 61 (Fig. 2). The mass spectrum of B had an ion at *m/z* 208 (M^+-60), suggesting the compound was a pentadecenyl acetate. DMDS derivatization produced an adduct with a molecular ion at *m/z* 362 (Fig. 2). Ions were observed at *m/z* 131 [$CH_3(CH_2)_4CH(SCH_3)^+$] and 231 (M^+-131), suggesting that compound B was Δ9-pentadecenyl acetate. Synthesis of 15:OAc, Z9-15:OAc, and E9-15:OAc, and comparison of retention times and mass spectra with those of compounds A and B confirmed them to be 15:OAc and Z9-15:OAc,

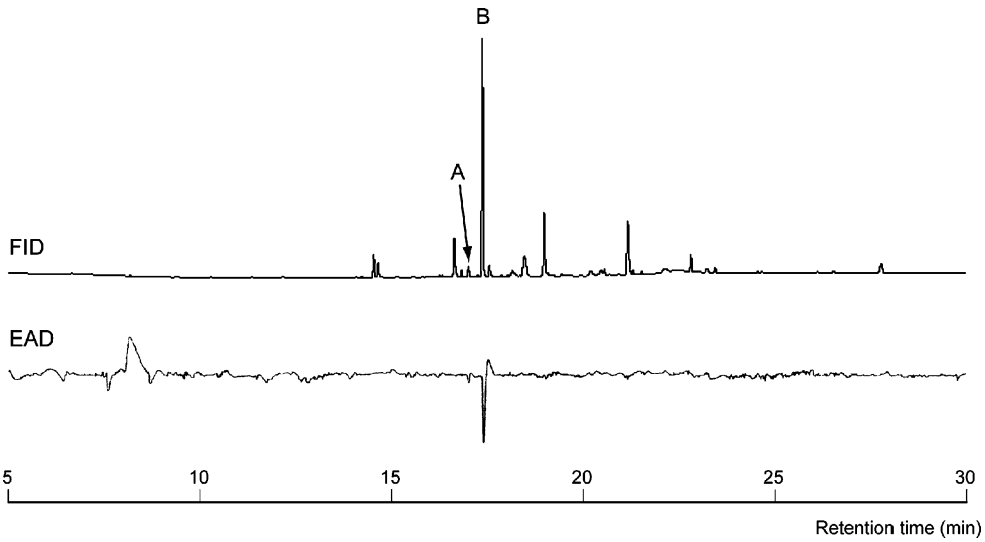

Fig. 1 Coupled gas chromatogram-flame ionization detection (*FID*; *upper trace*) and electroantennogram detection (*EAD*; *lower trace*) responses to a crude extract (ca. one female equivalent) of sex pheromone gland of *A. pyrivorella*. Compound A was subsequently identified as pentadecyl acetate and compound B as (*Z*)-9-pentadecenyl acetate

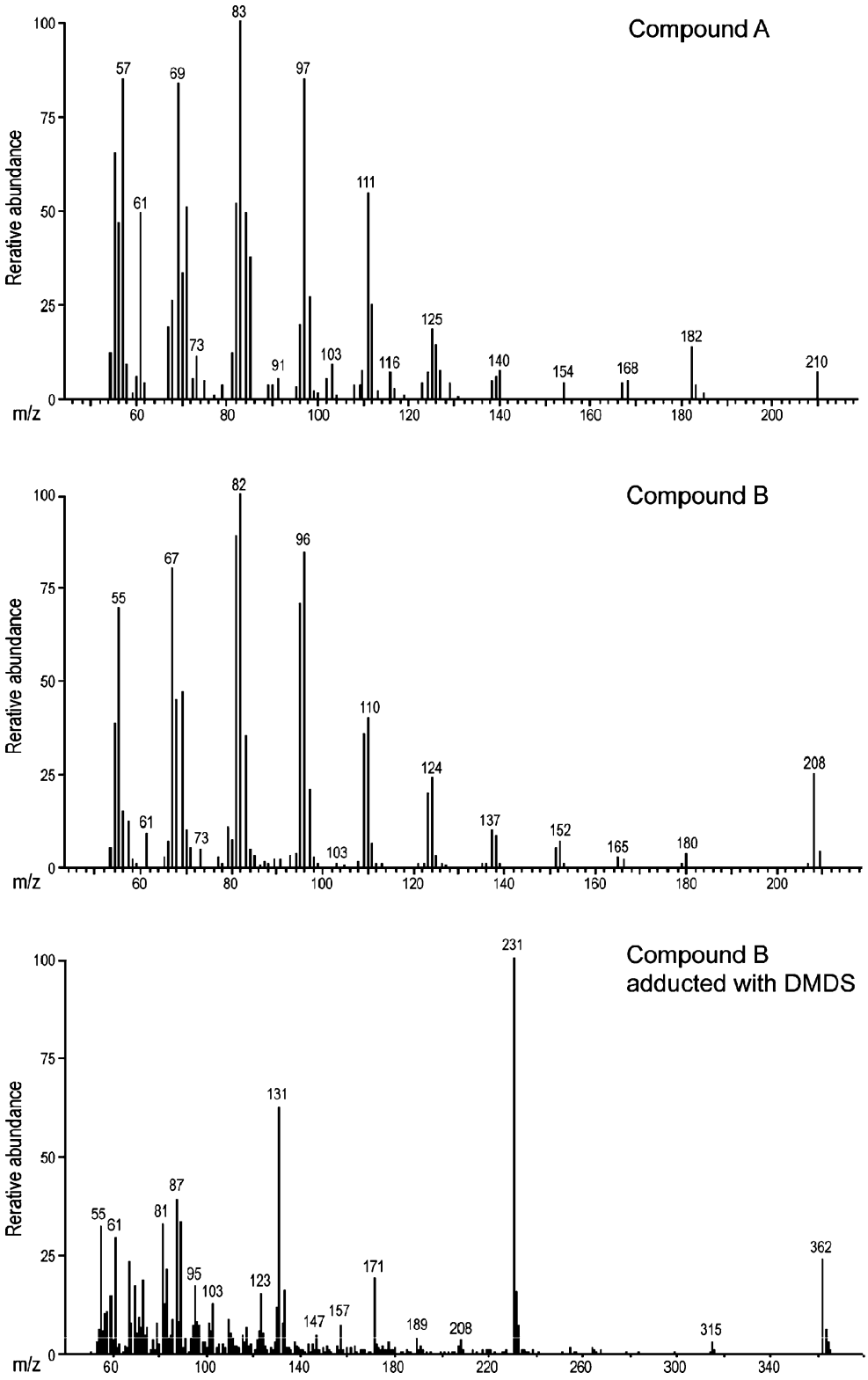

Fig. 2 Electron impact mass spectra of compound A (pentadecyl acetate), compound B [(*Z*)-9-pentadecenyl acetate], and the DMDS adduct of compound B

respectively (Table 1). The difference between the retention time of authentic Z9-15:OAc and E9-15:OAc was small, but the retention times of the respective DMDS adducts were resolved clearly (Table 1).

Male Attraction to Synthetic Pheromone Components in the Field In the first field trapping experiment, we tested the efficacy of the two compounds. Although the trap baited with 300 μg Z9-15:OAc caught males (total of 27), the trap

Table 1 Retention times (min) of EAD-active and authentic chemicals, and their DMDS adducts, on polar (DB-wax) and apolar (HP-1) columns

Column	EAD-active compound			Authentic compound[a]				
	A	B	DMDS adduct of B	15:Ac	Z9-15:OAc	E9-15:OAc	DMDS adduct of Z9-15:OAc	DMDS adduct of E9-15:OAc
DB-wax	20.66	21.11	46.47	20.66	21.11	21.06	46.47	47.53
HP-1	16.17	15.85	23.46	16.17	15.85	15.91	23.46	23.48

[a] 15:OAc: pentadecyl acetate; Z9-15:OAc: (*Z*)-9-pentadecenyl acetate; E9-15:OAc: (*E*)-9-pentadecenyl acetate.

baited with 300 μg Z9-15:OAc + 21 μg 15:OAc (7% of Z9–15:OAc quantity, the same ratio as in the extracts) caught (total of 45) significantly more (two-way ANOVA: for treatment, F=5.78, df=1, P=0.043; for block effect, F=1.77, df=8, P=0.21) (Fig. 3a). No males were caught in the trap with the lure containing 21 μg 15:OAc or in the control trap.

Experiment 2 examined the effect of E9-15:OAc on the attractive binary blend. Traps baited with Z9-15:OAc (300 μg) + 15:OAc (21 μg) + E9-15:OAc (30 μg; 10% of Z9-15:OAc) captured slightly more (30 in total) males than traps without E9-15:OAc (24), although the difference was not significant (two-way ANOVA: for treatment, F=0.005, df=1, P=0.95; for block effect, F=2.26, df=5, P=0.20).

Experiment 3 compared the attractive binary blend of Z9–15:OAc + 15:OAc with virgin females. Traps baited with the binary blend captured significantly (two-way ANOVA: for treatment, F=11.6, df=1, P=0.008; for block effect, F=1.13, df=9, P=0.87) more males (24 vs 2) than did traps baited with virgin females.

Further experiments determined the optimal ratio and dose of Z9-15:OAc and 15:OAc for attracting *A. pyrivorella* males. Experiment 4 varied the amount of 15:OAc (10.5, 21, or 42 μg) relative to Z9-15:OAc (300 μg). No significant differences were found among treatments (two-way ANOVA: for treatment, F=2.31, df=2, P=0.11; for block effect, F=1.14, df=23, P=0.35; Fig. 3b). Experiment 5 tested lures containing 100, 300, and 1,000 μg of Z9-15:OAc with 7% 15:OAc. Traps baited with a lure with 1,000 μg Z9-15:OAc captured more males than those containing 100 g or 300 μg (two-way ANOVA: for treatment, F=16.8, df=2, P<0.001; for block effect, F=1.18, df=23, P=0.31; Tukey–Kramer HSD test, α=0.05; Fig 3c). Experiment 6 tested higher concentrations: 1,000, 3,000, and 10,000 μg of Z9-15:OAc with 7% 15:OAc. Traps baited with either 3,000 or 10,000 μg Z9-15:OAc caught more males than traps with 1,000 μg (two-way ANOVA: for treatment, F=11.9, df=2, P<0.001; for block effect, F=1.80, df=23, P=0.044; Tukey–Kramer HSD test, α=0.05; Fig. 3d). No males were caught in the control (solvent only) traps in any of these experiments.

Taxonomic Reinvestigation of A. pyrivorella Roesler (1985, 1987) revised the generic status of the pear-fruit moth from *Ectomyelois pyrivorella* (Matsumura) to *Acrobasis pyrivorella*

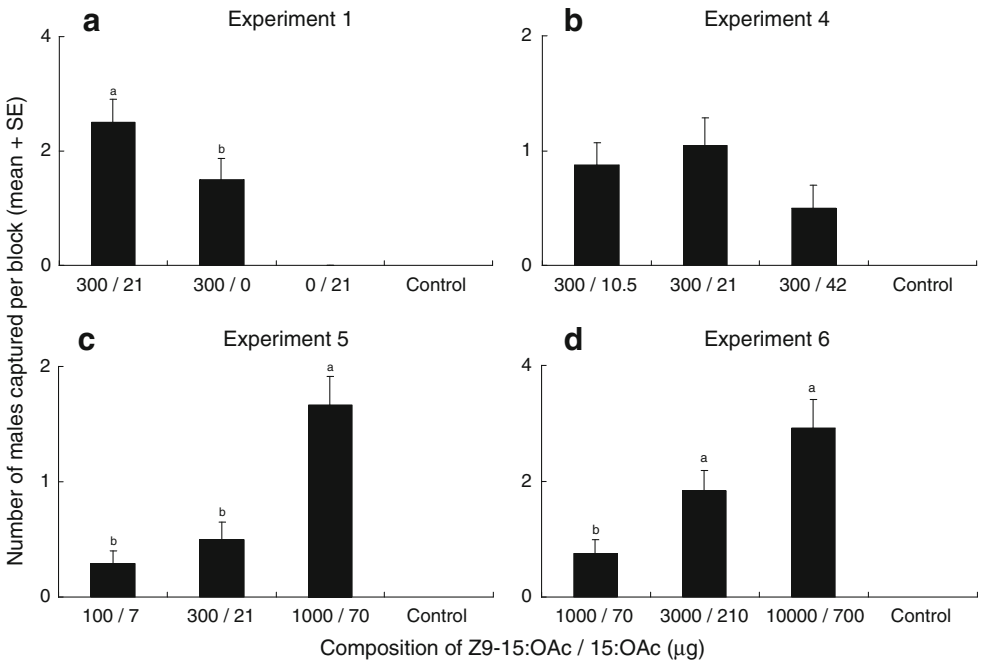

Fig. 3 Mean numbers (±SEM) of *A. pyrivorella* males captured (per block) in field experiments testing different ratios and amounts of candidate sex pheromone components, Z9-15:OAc [(*Z*)-9-pentadecenyl acetate] and 15:OAc (pentadecyl acetate). Control = solvent blank. Means with different *letters* atop bars in each experiment were significantly different by two-way ANOVA followed by the Tukey–Kramer HSD test (P<0.05)

(Matsumura). However, Japanese taxonomists have not agreed on this change, and the species is still referred to as *Ectomyelois pirivorella* in the recent list of Japanese moths (Jinbo, 2004–2008) after Inoue (1982). We therefore re-examined the morphological characters of adult *A. pyrivorella* to determine which genus it should be assigned to. Our results support assignment as *Acrobasis*: vein M2 stalked with M3 for shorter distance in hindwing (in *Ectomyelois* species M2–M3 stalked for nearly half the length); male genitalia with thicker phallus, and apical process of gnathos narrowly rounded (in *Ectomyelois*, more or less bifurcated at apical process) (Fig. 4). Other genital characters were similar to other *Acrobasis* species, including *Acrobasis consociella* (= *Acrobasis tumidella*, type species), drawn and described by Heinrich (1956).

Discussion

Z9-15:OAc and 15:OAc were identified in the pheromone gland of female *A. pyrivorella*, with Z9-15:OAc being more abundant. A lure containing only Z9-15:OAc (300 μg) attracted males in the field, but a blend of this compound with 15:OAc (21 μg; 7% of Z9-15:OAc) was significantly more attractive. Traps baited with these two chemicals caught more males than ones baited with two virgin females. We, therefore, conclude that Z9-15:OAc and 15:OAc are sex pheromone components of *A. pyrivorella*. Although changing the ratio of these two compounds (with both chemicals present) had little effect on trap catch, increasing the total amount of the compounds did, with blends containing 10,000 or 3,000 μg of Z9-15:OAc (+ 7% 15:OAc) eliciting the greatest catches of males. This blend could be used in traps to monitor for the emergence of *A. pyrivorella* or, indeed, in mating disruption leading to an efficient management program for this pest in pear orchards.

In several moth species, the geometric isomer of the pheromone component acts as an antagonist for the attraction of males. For example, the addition of more than 0.3% (*E*)-9-tetradecenyl acetate inhibited the attractiveness of the two-component mixture of (*Z*)-9- and (*Z*)-11-tetradecenyl acetate to the summer fruit tortrix *Adoxophyes orana fasciata* Walsingham (Sugie et al. 1984). In the case of *A. pyrivorella*, however, the attractiveness of the Z9-15:OAc + 15:OAc mixture was not significantly decreased by the addition of 10% E9-15:OAc (which was not detected in the pheromone gland). Hence, it is not necessary to remove small amounts of *E* isomer from synthetic Z9-15:OAc in order to use the latter as an attractant for *A. pyrivorella*. This is economically favorable for the development of the pheromone as a pest management tool.

Most moth pheromone components are even-numbered straight-chain acetates, alcohols, or aldehydes (Tamaki 1985); odd-numbered straight-chain compounds such as Z9-15:OAc and 15:OAc are rare (Witzgall et al. 2004). To our knowledge, ours is the first study to identify Z9-15:OAc and 15:OAc as true sex pheromone components of a moth. Z9-15:OAc and 15:OAc were found in the pheromone gland of a related *Acrobasis* species, the cranberry fruit worm *A. vaccinii* Riley, although neither compound had significant pheromonal activity in behavioral trials; the known pheromone blend of *A. vaccinii* consists of a mixture of (*E*,*Z*)-8,10-pentadecadienyl acetate and E9-15:OAc (McDonough et al. 1994). A few males of another *Acrobasis* species, *A. rufilimbalis* (Wileman), were captured in traps baited with Z9-15:OAc in field-screening tests of various compounds (unsaturated C_{10}–C_{16} straight-chain compounds containing a terminal hydroxyl or acetate

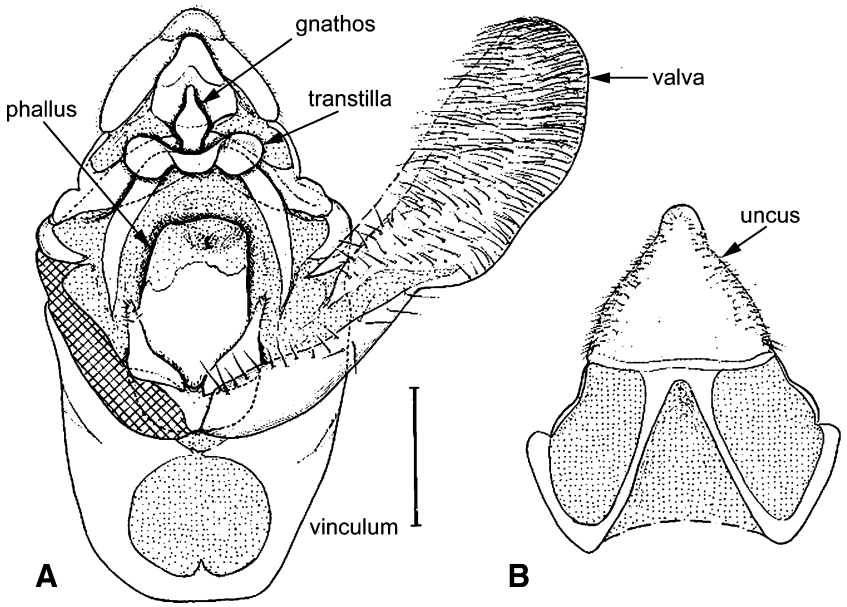

Fig. 4 Male genitalia of *A. pyrivorella*. **A** Ventral view, left valva removed; **B** dorsal view, tegumen and uncus. *Scale*: 0.5 mm

group; Ando et al. 1977). However, to date, pheromone chemicals produced by females have not been investigated in this species. Also, (*E*,*Z*)-9,11-pentadecadienal was slightly attractive to males of *A. nuxvorella* Neunzig, which produces the homologous (*E*,*Z*)-9,11-hexadecadienal as its true pheromone component (Millar et al. 1996).

In accordance with our morphological investigation, the sex pheromone components of *A. pyrivorella* are consistent with its assignment to the genus *Acrobasis* rather than *Ectomyelois*. As discussed above, C_{15} compounds are found in, or attract, other species of *Acrobasis*. The only species of *Ectomyelois* for which sex pheromone components have been identified is the carob moth, *Ectomyelois ceratoniae* (Zeller), which uses a blend of C_{14} aldehydes (*Z*,*E*)-9,11,13-tetradecatrienal, (*Z*,*E*)-9,11-tetradecadienal, and (*Z*)-9-tetradecenal (Baker et al. 1991). Thus, our combined findings support the taxonomic placement of the pear fruit moth in the genus *Acrobasis*, not *Ectomyelois*. The specimens of the pear fruit moth that we examined are deposited in the Laboratory of Applied Entomology, Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, for future taxonomic investigation.

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Chemical Composition of the Defensive Secretion of the Longhorned Beetle, *Chloridolum loochooanum*

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Abstract Adults of the longhorned beetle, *Chloridolum loochooanum* Gressitt (Coleoptera: Cerambycidae) emit a white frothy secretion from their metasternal glands. This defensive substance contains cyclopentanoid monoterpenoids (iridodials), whose structures were elucidated by gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) analyses that compared the naturally occurring structures with synthesized versions. Optically active citronellals, [(*S*)-, (*R*)-, and (*S*)/(*R*)- mixture], were used as starting materials for synthesizing the corresponding iridodials for the determination of the absolute configuration of the natural product. The retention time of (2*S*)-iridodial, derived from (*S*)-citronellal, corresponded to that of *C. loochooanum* iridodial by enantioselective GC analysis. Thus, we suggest that the absolute configuration of *C. loochooanum* iridodial is (1*R*,2*S*,5*S*)-iridodial.

Keywords Cerambycidae · Chemical defense · Coleoptera · Iridodials · Longhorned beetle · NMR

Introduction

Various strategies such as chemical defense, surface hardness, and/or mimicry have evolved that protect insects from predators. Defensive chemicals have been investigated frequently because of their diversity and species-specificity. Most defensive chemicals in beetles (Coleoptera) are odoriferous and repugnant to humans, e.g., formic acid, benzoquinones, cresols. (Eisner et al. 1977; Markarian et al. 1978; Balestrazzi et al. 1985; Kanehisa and Kawazu 1985). However, when attacked by predators, longhorned beetles in the genus *Chloridolum* are known for emitting a fragrant odor (recognizable to humans) from metasternal glands. Other defensive chemical secretions from cerambycid beetles include rose oxide and iridodials from the European musk beetle, *Aromia moschata* L. (Vidari et al. 1973); a strong but not unpleasant secretion consisting mainly of 2-hydroxy-6-methyl-benzaldehyde from the Eucalyptus longhorned borer, *Phoracantha semipunctata* (F.) (Moore and Brown 1972); and macrocyclic lactones, such as decan-9-olide, (*Z*)-dec-4-en-9-olide, and 11-hydroxytetradec-5-en-13-olide, from *Phoracantha synonyma* (Moore and Brown 1972, 1976; reviewed by Allison et al. 2004).

The longhorned beetle, *Chloridolum loochooanum* Gressit (Cerambycidae), occurs only on the islands of Amami and Okinawa in Japan (Gressitt 1933; Kojima and Hayashi 1969). *C. loochooanum* emits a delicate scent during flight (Makihara and Ohmura 2005). We were interested in the chemical composition of the defensive secretion of *C. loochooanum*, and used gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) to determine the chemical structures.

Methods and Materials

Insects Fallen *Viburnum awabuki* K. Koch infested with *C. loochooanum* (Makihara 2005; Fig. 1) were collected in March and April, 2003 on Okinawa island and were sent

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Fig. 1 An adult of *C. loochooanum* Gressit (Coleoptera: Cerambycidae)

immediately to the Forestry and Forest Products Research Institute (FFPRI), Tsukuba, Japan. They were maintained at 25°C until adult *C. loochooanum* emerged. Freshly emerged adults were sexed, and the defensive secretion was collected in the laboratory.

Collecting the Defensive Secretion When disturbed, *C. loochooanum* adults promptly emit a white liquid secretion from their metathoracic glands. The emitted secretion was absorbed on glass wool, which was extracted with $CDCl_3$ (Acros Organics, NJ, USA), or the secretion was absorbed directly on a solid phase microextraction (SPME) fiber (100 μm PDMS, Supelco, Tokyo, Japan) at room temperature in the laboratory.

Synthesis of Iridodials Iridodials were synthesized according to Clark et al. (1959) with a few further amendments. All unspecified reagents were purchased from Wako (Tokyo, Japan). A 3:1 mixture of (*S*)/(*R*)-citronellal [i.e., 75%-(*S*):25%-(*R*)] [(*S*)-(−)-citronellal, 96%, $[\alpha]_D^{25} = -17.3°$ (C=3, $CHCl_3$); (*R*)-(+)-citronellal, 90%, $[\alpha]_D^{25} = +14.0°$= (C=3, $CHCl_3$)] (Sigma-Aldrich, Tokyo, Japan; 25 ml), ethyleneglycol (15 ml), *p*-toluene sulfonate (30 mg), and benzene (240 ml) was refluxed in a Dean-Stark apparatus for 24 h to give the cyclic acetal in 83% yield. Selenium oxide, suspended in 50 ml of EtOH was added to the acetal in 30 ml of EtOH under reflux at 100°C for 20 h. The resulting aldehydic cyclic acetal (71% yield) was hydrolyzed and cyclized by refluxing with 50% acetic acid under Ar for 4 h. The reaction mixture was purified with silica-gel column chromatography (Silica-gel 60, 230–400 mesh, ASTM, Merck KGaA, Darmstadi, Germany) with *n*-hexane/EtOAc=4/1 as solvent. The fraction containing iridodials was re-chromatographed with CH_2Cl_2/acetone= 50/1 to obtain iridodials (0.13% yield).

Conversion of Iridodial to Nepetalinic Acid Dimethylester A compound whose chemical shifts coincided with those of *C. loochooanum* iridodial was subjected to oxidation by using pyridinium dichromate in dry dimethylformamide to form the methyl ester (O'Connor and Just 1987), in order to determine the relative configuration of *C. loochooanum* iridodial.

Chemical Analysis Capillary GC-MS analyses were carried out on a Shimadzu GC-17B system equipped with Shimadzu QP-5000 MS detector (Shimadzu Corporation, Kyoto, Japan), and a Hewlett Packard HP-6890 GC system interfaced with an HP-5973 MS detector (Agilent Technologies, Tokyo, Japan) under the following conditions: column (1) DB-1 on GC-17B (15 m×0.25 mm, film thickness 0.25 μm, J & W Scientific, Folsom, CA, USA), injection port temperature, 250°C, transfer line, 280°C, carrier gas, helium (flow, 1 ml/min), ionization voltage, 70 eV. The column temperature was kept at 50°C for 1 min, 50°C to 200°C (5°C/min); column (2) HP-5 on HP-6890 (30 m×0.25°mm, film thickness 0.25 μm, J & W Scientific), injection port temperature, 240°C, transfer line, 260°C, carrier gas, helium (flow, 1 ml/min), ionization voltage, 70 eV. The column temperature was kept at 40°C for 1 min, 40°C to 250°C (5°C/min); and column (3) β DEX 225 on HP-6890 (30m×0.25 mm, film thickness 0.25 μm, Supelco, Tokyo, Japan), injection port temperature 190°C, transfer line, 230°C, carrier gas, helium (flow, 1 ml/min). The column temperature was kept at 40°C for 1 min, 40°C to 199°C (3°C/min). When analyzing chemicals absorbed on SPME fibers on the DB-1 column, samples were introduced at 250°C via a specialized inlet liner (0.75 mm I.D., Supelco) in the injection port of the GC-MS. Samples were desorbed from the fibers for 1 min (splitless mode, 1 ml/min), and fibers were re-conditioned at 250°C for 30 min. NMR spectra were measured with JEOL LAMBDA-400 and ALPHA-500 spectrometers (JEOL, Tokyo, Japan). $CDCl_3$ was used as the solvent.

Results

A major peak and three smaller peaks were detected in the secretory volatiles from male and female *C. loochooanum* (Fig. 2). These peaks, which we assumed to be isomers of one another, were present in samples extracted both by SPME and methylene chloride. GC-MS analysis revealed similar fragmentation patterns and also the same molecular ion (*m/z* 168) for all of these peaks (Fig. 3). The diagnostic fragment ions of each peak were *m/z*: 150, 135, 111 or 109, 93, 81, and 67. Fragment ions at *m/z* 150 (M^+–18) and 135 (M^+–18–15) suggested an unstable oxygenated group and a methyl branch in the molecule. The base peak (*m/z* 81) has been observed typically in the fragment patterns of a number of iridoid monoterpenes (Regner 1972).

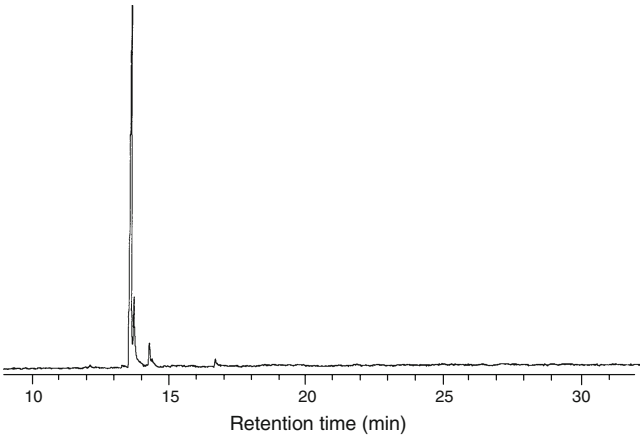

Fig. 2 Total ion chromatogram of the defensive secretion of *C. loochooanum* desorbed from an SPME fiber and analyzed with a DB-1 capillary column (15 m×0.25 mm, film thickness 0.25 μm) on a Shimadzu GC-17B instrument

The ^{1}H-NMR spectrum of the major component showed signals for 16 protons including two aldehydic protons (δ= 9.59, d, J=3.7 Hz; δ=9.63, d, J=2.4 Hz) and six methylic protons (δ=1.079, d, J=6.7 Hz; δ=1.085, d, J=7.3 Hz). No NMR analysis of the minor components was possible because of the low amount of available natural product. These chemical analyses indicated that the components were dialdehydes of cyclopentanoid monoterpenoids, i.e., various forms of iridodial.

The coupling constants of $J_{1,2}$ and $J_{1,5}$ were both 8.5 Hz, suggesting the *trans*,*trans* configuration, although some difficulties have been reported in determining the relative stereochemistry of a cyclopentanoid skeleton (Constantino and da Silva 1998). In order to determine the relative configuration of the primary defensive chemical of *C. loochooanum*, iridodials were synthesized according to Clark et al. (1959) (Fig. 4). After fractionation by silica-gel column chromatography, a compound whose chemical shifts coincided with those of the defensive secretion was subjected to oxidation according to O'Connor and Just (1987). By comparing ^{1}H- and ^{13}C-NMR spectral data of

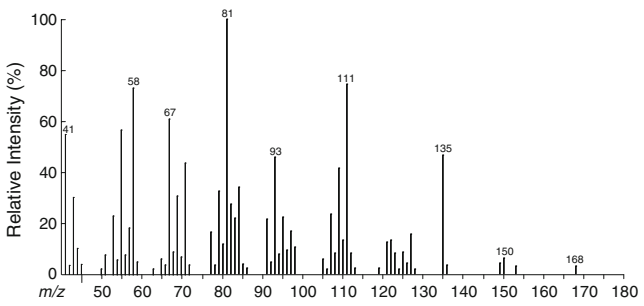

Fig. 3 Mass spectrum of the main compound in the defensive secretion of *C. loochooanum*

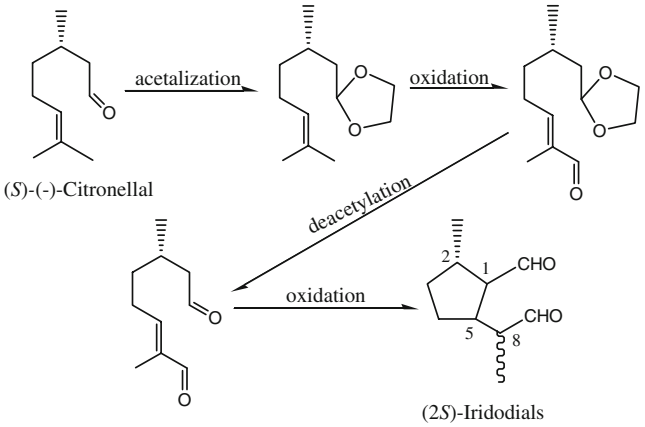

Fig. 4 Synthesis scheme of (2*S*)-iridodials from (*S*)-(−)-citronellal (Clark et al. 1959)

the resulting nepetalinic acid methyl ester with those of Sakai et al. (1981), the relative configuration of *C. loochooanum* iridodial was determined as *trans*,*trans*. According to Oldham (1994), the configuration at the 2-position in natural iridoids is exclusively (*S*)-, however, (2*R*)-isomers, e.g., boschnaloside (8-*epi*-iridodial glucoside; Murai and Tagawa 1982) and 8-*epi*-loganic acid and gmephiloside (Helfrich and Rimpler 2000) also have been reported.

The absolute configuration of *C. loochooanum* iridodial was confirmed by synthesis and GC-MS analysis. Different enantiomeric blends of citronellal, (*S*)-, (*R*)-, and a 3:1 mixture of (*S*)/(*R*), were used as the starting materials for synthesizing the corresponding iridodials. Both the defensive secretion of *C. loochooanum* and synthesized iridodials were analyzed with an enantioselective GC column by GC-MS. *C. loochooanum* iridodial coincided with iridodial synthesized from (*S*)-citronellal, but not with the one synthesized from (*R*)-citronellal (Fig. 5).

(1R,2S,5S)-Iridodial (from C. loochooanum) ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.079 (3 H, d, J=6.7 Hz, H-7), 1.085 (3 H, d, J=7.3 Hz, H-10), 1.35 (1 H, m, H-3), 1.90 (2 H, m, H-3, H-4), 1.95 (1 H, m, H-4), 2.11 (1 H, t, J=3.7, 8.5, 8.5 Hz, H-1), 2.15 (1 H, m, H-2), 2.4 (1 H, t, J=1.8, 7.7, 7.7 Hz, H-8), 2.6 (1 H, m, H-5), 9.59 (1 H, d, J= 3.7 Hz, H-6), 9.63 (1 H, d, J=2.4 Hz, H-9). GCMS M+ 168.0 (3%), *m/z* 153.1 (3%), 150.1 (6%), 135.2 (47%), 111.2 (74%), 109.1 (41%), 93.1 (46%), 81.2 (100%), 67.2 (61%).

trans,trans-Nepetalinic acid dimethylester ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.03 (3H, d, J=6.3 Hz, H-7), 1.12 (3 H, d, J=7.1 Hz, H-10), 1.25 (1 H, m, H-3), 1.47 (1 H, m, H-4), 1.86 (2 H, m, H-3, H-4), 2.08 (1 H, m, H-1), 2.15 (1 H, m, H-2), 2.45 (1 H, m, H-8), 2.55 (1 H, m, H-5), 3.66 (3 H, s, $COOCH_3$), 3.69 (3 H s, $COOCH_3$); ^{13}C NMR

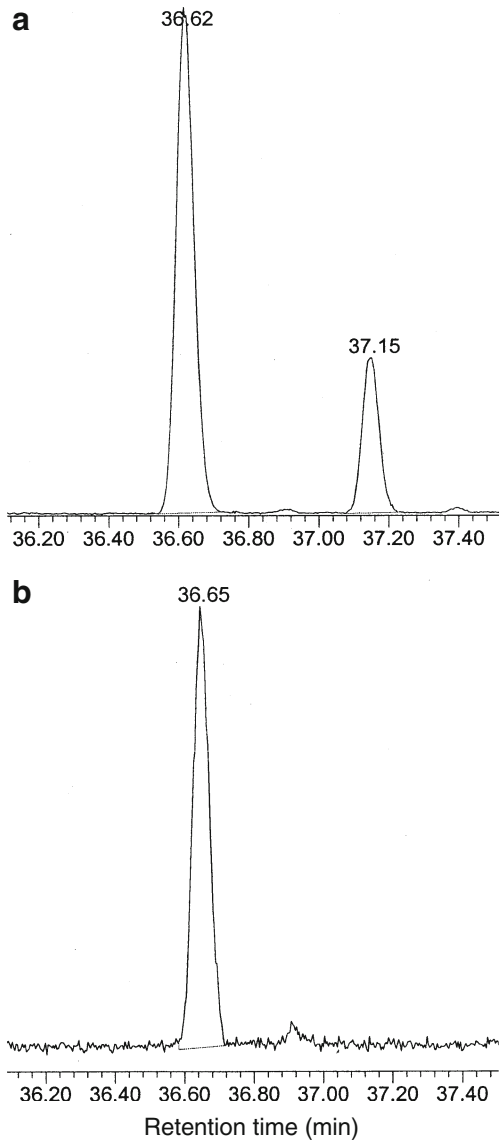

Fig. 5 Enantioselective GC chromatograms of **a** iridodials synthesized from (*S*)/(*R*)-citronellal and **b** naturally occurring iridodial from *Chloridolum loochooanum*

($CDCl_3$) δ 15.2 (C-10), 19.4 (C-7), 28.8 (C-4), 33.5 (C-3), 40.7 (C-2), 43.8(C-8), 46.6 (C-5), 51.46($COO\underline{C}H_3$), 51.50 ($COO\underline{C}H_3$), 56.1(C-1), 176.0 ($\underline{C}OOCH_3$), 176.5 ($\underline{C}OOCH_3$).

Discussion

Chemical ecological studies on longhorned beetles, as pests in forests, log yards, and urban settings, have advanced our knowledge of the chemical structures and biological activities of their attractants and pheromones (Fukaya et al. 1996; Allison et al. 2004). Although some cerambycids also emit defensive semiochemicals from metasternal glands (Moore and Brown 1972, 1976; Vidari et al. 1973), little is known about their function in relation to predators (Allison et al. 2004). The defensive strategies of cerambycid beetles vary among genera. Body colors are basically dark, suggesting that color mimicry of tree bark or leaves might help them hide from enemies. Some diurnally active cerambycids have aposematic coloration that mimics that of vespids (wasps), and thus protects them from predators, especially birds. For example, *Chlorophorus quinquefasciatus* (C. and G.) in the Ryukyu Islands exhibits geographic variation of its spot pattern, which coincides with vespid distribution in their habitat (Makihara 2003). In the tribe Callichromatini, *Pachytenia* sp., having a distinct two-tone color pattern, is thought to defend itself by mimicking vespids (e.g., *Vespa affinis*), whereas *Aroma moschata* (Vidari et al. 1973) and *Chloridolum* sp. are known to emit chemicals against their predators.

Our results revealed that (1*R*,2*S*,5*S*)-iridodial was secreted from the metasternal glands of the longhorned beetle, *C. loochooanum*. Iridodials are well known as defensive chemicals in many insects, e.g., the longhorned beetle, *A. moschata* (Vidari et al. 1973), ants (McGurk et al. 1968; Cavill and Houghton 1974; Attygalle and Morgan 1984; Do Nascimento et al. 1998), the stick insect, *Graeffea crouani* (Smith et al. 1979), rove beetles (Huth and Dettner 1990; Weibel et al. 2001), and leaf beetles (Burse et al. 2007). Among iridodials, *trans*,*trans*-iridodial was reported as a defensive component in the coconut stick insect, *G. crouani* (Smith et al. 1979), and in the dolichoderine ants, *Iridomyrmex* spp. (McGurk et al. 1968) and *Tapinoma nigerrium* (Oldham 1994). Structurally related iridoid monoterpenes, such as peruphasomal (Dossey et al. 2008), dolichodial (Cavill and Hinterberger 1961), anisomorphal (Meinwald et al. 1962), nepetalactone (Smith et al. 1979), chrysomelidial, and plagiolactone (Meinwald et al. 1977), also have been reported as defensive chemicals in insects. Some of these chemicals, including iridodials, have been demonstrated to have other biological activities, i.e., as attractive pheromones or interspecific repellents (allomones). In lacewings, (1*R*,2*S*,5*R*,8*R*)-iridodial has been identified as a male-produced pheromone in *Chrysopa oculata* (Chauhan et al. 2004; Zhang et al. 2004), *Chrysopa nigricornis* (Zhang et al. 2006a), and *Chrysopa septempunctata* (Zhang et al. 2006b). (4a*S*,7*S*,7a*R*)-Nepetalactone and related nepetalactols have been reported to be sex pheromones of aphids (Birkett and Pickett 2003). A silver vine, *Actinidia polygama*, produces *trans*,*cis*- and *cis,trans*-iridodials in leaves and fruits as an oviposition attractant for a gall midge, *Asphondylia matatabi*, and also produces isoiridomyrmecin, isodihydronepetalactone, and 8,9-isodehydroiridomyrmecin that are attractants and sedatives for felids (Murai and Tagawa 1994, 1997). Dolichodial and

teucrein from a cat thyme, *Teucrium marum* (Eisner et al. 2000), and nepetalactones and elemol from a catnip, *Nepeta cataria* (Schultz et al. 2004), have repellent activities for ants and house flies.

Defensive chemicals in prey are considered to cause some physiological damage to predators, and thus allow prey to escape from attack. The presence of two aldehyde groups in iridodial and related chemicals indicates high reactivity and toxicity to living cells. Various iridoidglycosides exist in plants in nonpoisonous forms derived from their aglycones, iridoid monoterpenes, which are transformed to their active forms by hydrolysis with glucosidase (Murai et al. 1987) or acetyl-transferase (Eisner et al. 2000). In insects, volatile terpenoids are stored in and emitted from glands that have cuticle layers, which are isolated from other organs (Chapman 1998). They are biosynthesized and/or sequestered from plant allelochemicals, and used for their defense (Laurent et al. 2005).

Similarly, we suggest that adult *C. loochooanum* may retain iridodial in their glands and use it during predator attacks, as residual iridodial may cause severe injury to predators. The beetle also may have other nonvolatile defenses in its body that make itself more unpalatable. Further research will be necessary to answer these ecological questions.

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Effects of Soldier-Derived Terpenes on Soldier Caste Differentiation in the Termite *Reticulitermes flavipes*

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Abstract Primer pheromones play key roles in regulating division of labor, which is a fundamental and defining aspect of insect sociality. Primer pheromones are chemical messengers that transmit hormone-like messages among colony members; in recipients, these messages can either induce or suppress phenotypic caste differentiation. Here, we investigated soldier caste-derived chemicals as possible primer pheromones in the lower termite *Reticulitermes flavipes*, a species for which no primer pheromones have yet been identified. We determined that soldier head extracts (SHE), when provided to totipotent workers along with the insect morphogenetic juvenile hormone (JH), significantly enhanced soldier caste differentiation. When applied alone, however, SHE had no impacts on caste differentiation, survivorship, or any other aspect of worker biology. These findings support a function of soldier chemicals as primer pheromones that enhance the action of the endogenous JH. In accord with previous studies, γ-cadinene and the corresponding aldehyde, γ-cadinenal, were identified by gas chromatography–mass spectrometry and nuclear magnetic resonance analyses as the two most abundant components of *R. flavipes* SHE. Validative bioassays with commercially available cadinene confirmed activity. Several other terpenes, previously identified in *R. flavipes* soldiers, also were found to be active. These findings reveal a novel primer pheromone-like function for soldier-derived terpenes in termites and further suggest convergent evolution of terpene functions in enhancing JH-dependent soldier caste differentiation.

Keywords Termite · Soldier · Primer pheromone · Juvenile hormone · Terpene

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Introduction

Social insect castes are groups of phenotypically, morphologically, and behaviorally distinct individuals that cooperate to perform colony tasks (Wilson 1971; Miura 2004). Caste differentiation plays an important and necessary role in creating an effective division of labor. Colonies must find ways to regulate caste differentiation within this system, as improper regulation could result in the overabundance or absence of specific castes, thus making colony tasks such as food acquisition, grooming, defense, and reproduction inefficient or impossible.

Polyphenisms are alternative morphological phenotypes that differentiate in response to environmental conditions (Nijhout 2003). Termites use polyphenism to produce different castes that perform complementary roles within the colony (Miura 2004). Termite colonies are made up of three distinct castes that include workers/pseudergates, soldiers, and reproductives. Only soldiers and reproductives are considered adults in lower termites, while all castes can

be adults in higher termites. Termite caste differentiation can proceed along two routes: the imaginal (winged) or the apterous (wingless) route. The first developmental branch occurs when larvae differentiate into either workers or nymphs after the second instar (Buchli 1958; Lainé and Wright 2003). Workers can (1) undergo status quo, worker-to-worker molts; (2) differentiate into presoldiers (immediately followed by soldier differentiation); or (3) differentiate into apterous and eyeless third-form reproductives, or "ergatoid neotenics". Nymphs can (1) regress into worker-like pseudergates; (2) differentiate into fully winged and eyed adult alates that disperse, mate, and become primary reproductives; or (3) differentiate into wingless and eyed nondispersive, second-form reproductives, or "brachypterous neotenics" that serve as supplemental reproductives (Buchli 1958; Lainé and Wright 2003).

Caste polyphenism in social insects is distinct from polyphenism solitary insects because multiple castes that perform nonoverlapping tasks are present in colonies at the same time (Miura 2004). Individuals in termite colonies with the same genetic background can differentiate into alternate phenotypes depending on a number of intrinsic and extrinsic factors (Lenz 1976; Greenberg and Tobe 1984; Scharf et al. 2007). One intrinsic factor is juvenile hormone (JH; Scharf et al. 2003; Park and Raina 2004, 2005; Mao et al. 2005). JH is a morphogenetic hormone produced by a neurosecretory gland, the corpus allatum, that has a broad range of developmental and physiological effects (Truman and Riddiford 1999; Gilbert et al. 2000). For example, in insects, JH plays a role in the control of larval/nymphal development and metamorphosis, diapause, migratory behavior, wing length, seasonal development, reproduction, and caste determination (Hartfelder 2000).

Primer pheromones are chemical messengers that are passed among individuals and trigger physiological responses in recipients (Wilson and Bossert 1963). Primer pheromones are distinct from "releaser" pheromones, which elicit rapid behavioral responses in recipients (Vander Meer et al. 1998). Two examples of releaser pheromones in termites are the trail pheromone, (Z,Z,E)-3,6,8-dodecatrien-1-ol (Matsumura 1968), and the phagostimulatory pheromone, hydroquinone (Reinhard et al. 2002). Although no primer pheromones have been identified in termites, JH has been proposed as one (Henderson 1998). Previous studies have shown that ectopic exposure of worker termites to JH III readily induces soldier caste differentiation (Scharf et al. 2003, 2005, 2007; Zhou et al. 2006a, b, 2007), indicating that JH can act via exogenous exposure. Under natural conditions, high endogenous JH titers in worker termites cause differentiation into presoldiers and then into soldiers (Park and Raina 2004; Mao et al. 2005). Regardless of whether it acts exogenously as a primer pheromone, an endogenous hormone, or both, the role of JH in soldier development is unique and in contrast to the immature "status quo" role of JH among insects (Henderson 1998).

It has been hypothesized that termite soldiers may play a role in regulating worker differentiation to other caste phenotypes (Henderson 1998). For example, JH titers in workers rise upon removal from the colony (Okot-Kotber et al. 1993; Mao et al. 2005) and can result in presoldier/soldier formation (Mao et al. 2005). However, if workers are held with soldiers, worker JH titers remain below threshold levels, and presoldier formation is attenuated (Mao et al. 2005; Park and Raina 2005). It has been theorized that soldiers can downregulate worker JH titers by acting as a JH "sink" (Henderson 1998; Mao et al. 2005) or by removing the inhibitory effect of another primer pheromone on worker differentiation (Park and Raina 2004, 2005; Mao et al. 2005).

Previously, Lefeuve and Bordereau (1984) investigated live soldiers and the effects of dichloromethane (DCM) soldier head extracts (SHE) on caste differentiation in the higher termite *Nasutitermes lujae* and found that SHE inhibited worker-to-soldier differentiation. They suggested that soldier termites may secrete an inhibitory pheromone that contributes to worker–soldier homeostasis in termite societies. Korb et al. (2003) also reported that DCM SHE inhibited soldier formation in the lower termite *Cryptotermes secundus*. Additionally, Okot-Kotber et al. (1991) showed that soldier formation in *Reticulitermes flavipes* was reduced by DCM SHE when co-applied in combination with synthetic JH analogs. While these studies have verified primer pheromone-like effects for SHE, no *bona-fide* termite primer pheromones have yet been identified. Thus, two important questions in termite research relate to whether or not caste-regulatory primer pheromones exist, and if so, what are their chemical structures and modes of action?

R. flavipes and its European synonym *Reticulitermes santonensis* are common and economically destructive termites in the USA and Europe. The central objective of this study was to investigate chemical constituents of *R. flavipes* SHE as possible primer pheromones. To meet this objective, we conducted studies to (1) investigate SHE effects on JH-dependent soldier caste differentiation, (2) identify SHE constituents, and (3) compare constituent activity with previously identified soldier head chemicals. Through these studies, we provide evidence supporting the idea that soldier head terpenes play roles as caste-regulatory primer pheromones in termites.

Methods and Materials

Termites R. flavipes colonies were collected from various locations on the University of Florida campus. Termites were brought to the laboratory and held for at least 2 months

before use. Laboratory colonies were maintained in darkness within sealed plastic boxes at 22°C. A total of nine colonies were tested, all of which contained male and female neotenic reproductives. Termite workers were considered workers if they did not possess any sign of wing buds or distended abdomens. Termites were identified as *R. flavipes* from sequence of the 16S mitochondrial–ribosomal RNA gene, (Szalanski et al. 2003), gut fauna (Lewis and Forschler 2004), and soldier morphology (Nutting 1990).

Dish Assays Dish assays were conducted at 27°C as described previously (Scharf et al. 2003). Paired paper towel sandwiches were treated with control, JH III, or SHE treatments in acetone. JH III (75% purity; Sigma; St. Louis, MO, USA) was provided at 112.5 μg per dish in 200 μl acetone. This quantity was chosen based on its maximal efficacy and minimal mortality observed in previous studies (Scharf et al. 2003). SHE was tested at several different quantities (see below). After solvent evaporation, sandwiches were placed in 5-cm plastic Petri dishes and then each received 150 μl of reverse osmosis water. Fifteen worker termites were placed in each dish. Every 5 days, termites were counted, presoldier formation noted, and deionized water added, if needed.

Solider Head Extracts SHE was prepared by removing and homogenizing soldier heads (~80–150 total, depending on the experiment) in acetone with a Tenbroeck glass homogenizer. SHE was fractionated with acetone through a glass Pasteur pipette, filled with approximately 250 mg of silica gel (60–200 mesh). Acetone was added to each of the resultant ten fractions to make a final volume of 50 ml.

SHE Concentration Response and Investigation of Colony Variation SHE prepared in acetone was tested on three *R. flavipes* colonies (colonies 7, 8, and 9). Seven different treatments were tested: control (300 μl acetone), JH III (200 μl acetone containing 112.5 μg JH III), SHE alone (four head equivalents), and JH III plus a range of SHE equivalents (0.5, 1, 2, and 4). Each treatment was replicated six times.

Gas Chromatography and Mass Spectrometry Thirty soldier and worker heads from two different colonies (colonies 5 and 7) were extracted as described above, and the solvent volume was reduced to 400 μl under a stream of N_2. Samples were analyzed first by gas chromatography–mass spectrometry (GC–MS; electron ionization, 70 eV) to confirm the presence of the previously identified predominant terpenoids, γ-cadinene and γ-cadinenal (Nelson et al. 2001). These compounds were quantified subsequently by using a 6890 gas chromatograph (Agilent; Santa Clara, CA, USA) coupled to a flame ionization detector as described in Schmelz et al. (2001). We also examined pine wood extracts, prepared from the same "shim" wood used to provision lab colonies (seasoned and kiln-baked), to test the hypothesis that SHE chemicals are produced in termites de novo. Fresh pine wood sawdust (1.26 g) was extracted and analyzed as described above for SHE.

To quantify semiochemical amounts in individual soldier heads, five individual soldier heads were extracted as above. Individual extracts had a final volume of 400 μl with 400 ng of nonyl acetate added as an internal standard. Samples were analyzed by GC.

Nuclear Magnetic Resonance Analysis The two main peaks of the SHE were isolated by preparative GC and analyzed by nuclear magnetic resonance (NMR). Initial preparation of SHE utilized vapor phase extraction at 80°C on polymeric adsorbent traps, followed by DCM elution to remove less volatile contaminants (Schmelz et al. 2004). Micropreparative GC used an Agilent 6890 chromatograph (He carrier gas; 5.7 ml min^{-1}; cool on-column injector set to track oven) equipped with a DB-1 column (30 m× 530 μm i.d., 0.50 μm film thickness), and with the temperature programmed from 35°C (2 min hold) at 10°C min^{-1} to 260°C (hold for 5.5 min). Recovery of GC fractions was similar to that used by Heath and Dueben (1998) with the slight modification of a glass press-fit splitter at the end of the column, coupling a 0.5-m (150-μm i.d. fused silica) capillary to the flame ionization detector (FID) and a second 0.5-m (350-μm i.d. fused silica) capillary directed to the heated transfer line. A chilled glass capillary was used for sample collection. Under these conditions, the two predominant soldier head sesquiterpenes eluted at 16.1 and 18.9 min. An authentic standard of γ-cadinene eluted at 16.1 min.

One- (1-D) and two-dimensional (2-D) NMR spectra were acquired at 20°C with standard techniques using TopSpin® (version 2.1) software on a Bruker Avance-II-600 spectrometer equipped with a 1-mm high-temperature superconducting CryoProbe (Brey et al. 2006). Solutions of SHE γ-cadinene (~10 μg/15 μl), authentic γ-cadinene (~25 μg/17 μl), and SHE γ-cadinenal (50 μg/10 μl) were prepared in $CDCl_3$ (99.96 atom % D) and analyzed in 1 mm outer dimension×0.73 mm indirect dimension (ID)× 100 mm long capillary NMR tubes (Norell, Inc.). Proton spectra were acquired at 600.23 MHz with a 2.48-s acquisition time and a 3-s relaxation delay. The ^1H chemical shift axis was referenced to $CHCl_3$ and assigned to 7.26 ppm (Gottlieb et al. 1997). Two-dimensional $^1H/^1H$-COSY data sets (SW=8 ppm, AT=0.21 s, RD=2 s, two to eight transients) were acquired with Bruker's "cosygpqf" pulse sequence as 2048 complex points in the directly detected dimension (DD) and 512 increments in the ID and

processed with sine-function apodization into 1,024×1,024 point spectra. Carbon-13 spectra were acquired at 150.93 MHz. The ^{13}C chemical shift axis was referenced to $CDCl_3$, assigned to 77.16 ppm (Gottlieb et al. 1997). Multiplicity edited 2-D $^{1}H/^{13}C$-HSQC data sets (^{1}H SW= 8 ppm, ^{13}C SW=170 ppm, AT=0.14 s, RD=2 s, 48–96 transients) were acquired with Bruker's "hsqcedetg-psisp2.2" pulse sequence as 1,348 complex points in the DD dimension and 256 increments in the ID dimension and were apodized with cosine-squared functions into 2,048× 512 point spectra.

Testing of Previously Identified Chemicals Chemicals (or close structural analogs) were tested individually in dish assays on a single *R. flavipes* colony (colony 5). All treatments were applied at 50 μg/dish, with and without JH III (300 μl acetone containing 112.5 μg JH III). Individual chemical treatments were provided at a quantity equivalent to approximately 1/2 the JH III dose; this amount (50 μg/dish) approximates endogenous cadinene levels found in 25 soldier head equivalents, based on GC–MS analysis. Treatments were as follows: controls (in 300 μl acetone), SHE (four head equivalents in acetone), α-humulene (Aldrich, St. Louis, MO, USA), β-farnesene (Bedoukian, Danbury, CT, USA), cadinene (Vigon International, East Stroudsburg, PA, USA), geranyl linalool (Acros, NJ, USA), linalool (Aldrich), farnesol (Bedoukian), (+)β-pinene oxide (Acros), limonene (Aldrich), nootkatone (Bedoukian), nerolidol (Bedoukian), α-pinene (Acros), and geranylgeraniol (Sigma Aldrich). Control treatments were acetone, JH III, SHE, and JH III+SHE. Each treatment was replicated three times.

Statistical Analyses In all experiments, the number and caste of each termite in each dish was counted every 5 days. The percentage of presoldiers formed out of the total number of workers put into each assay was used for statistical analysis (Scharf et al. 2003, 2005; Zhou et al. 2006a, b). Data were first analyzed for normality using the *Levene* test. If not normal, data were transformed to ranked averages and means separated using the Tukey–Kramer test ($P<0.05$). For bioassays of previously identified soldier chemicals, ranked averages were separated using a least significant difference Student *t* test ($P<0.05$).

Results

SHE Concentration Response Three colonies were examined in SHE dose–response bioassays using SHE (Fig. 1). Two of the three colonies responded similarly, but one (colony 9) responded slightly differently, which led to a significant *colony* effect in the analysis of variance (df= 2,117, F=4.79, P=0.01). Nonetheless, a pooled dose–response analysis of the three colonies was conducted. Presoldier induction increased significantly when termite workers were co-exposed to SHE and JH III, as compared to treatments of JH III alone ($P<0.05$). Controls treated with either acetone or SHE alone resulted in no presoldier formation. Presoldiers first appeared between days 10 and 15 and reached maximum levels by day 25 in all SHE+JH III and JH III-alone treatments. This analysis verified that SHE does indeed cause a significant increase in presoldier formation when combined with JH III; however, this effect is not dose dependent in the range of 0.5–4 head equivalents (df=6,117, F=32.32, $P<0.001$). Data from additional experiments are provided in Fig. S1 and Table S1 (see Supplementary Material).

GC–MS and NMR Analysis GC–FID analyses of SHE identified two major sets of peaks (Fig. 2). Profiles of the peaks were similar to those reported by Zalkow et al. (1981) and Nelson et al. (2001), who identified γ-cadinene and γ-cadinenal as major whole-head extract components. The major peak in the first set was identified as γ-cadinene by comparing its mass spectrum (see Fig. S2, supplementary material), GC retention time and NMR spectra (1-D ^{1}H, 2-D $^{1}H/^{1}H$-COSY, and $^{1}H/^{13}C$-HSQC) with those of an authentic sample (kindly provided by Dr. Bartelt, USDA-ARS-NCAUR; Peoria, IL, USA) and literature values (Quintana et al. 2003).

The corresponding γ-cadinenal, the major peak of the second set of peaks, was identified by comparison of its ^{1}H NMR (see data below) and mass spectra (Fig. S2, supplementary material) to those reported by Kaiser and Lamparsky (1983). Since we observed some small differences between their 400 MHz ^{1}H spectrum and ours at 600 MHz, we also report the details of our ^{1}H NMR spectrum here, along with the 15 chemical shifts for the ^{13}C NMR resonances of the SHE γ-cadinene aldehyde.

NMR results are as follows ^{1}H: δ 9.47 (s, 1 H), 6.91 s, 1H), 4.74 ("d", J=1.5, 1 H), 4.62 ("d", J=1.3, 1 H), 2.52–2.46 (m, 1 H), 2.44 (ddd, J=2.9, 4.0, 13.0, 1 H), 2.26 (d septets, J=3.3, 6.9, 1 H), 2.15–2.07 (multiplets, 2 H), 2.06 (broad dt, J=4.5, 13.1, 1 H), 1.97–1.91 (t of "five-line patterns", 1H), 1.90–1.84 (multiplets, 2 H), 1.50–1.41 (multiplet, 1 H), 1.42 (tt, J=3.2, 11.6, 1 H), 1.21 (dq, J=4.2, 12.8, 1 H), 0.99 (d, J=6.9, 3 H), and 0.82 (d, J=6.9, 3 H). ^{13}C NMR results are as follows: δ 194.76, 151.78, 151.75, 141.77, 104.65, 46.37, 46.05, 44.07, 36.05, 26.69, 26.65, 24.45, 21.87, 21.57, and 15.34.

The average amount of γ-cadinene and γ-cadinenal from soldiers was 1.44±0.29 and 9.42±1.75 μg, respectively, with the amount of γ-cadinenal being significantly higher than the amount of γ-cadinene (df=1,8, F=20.29, P=

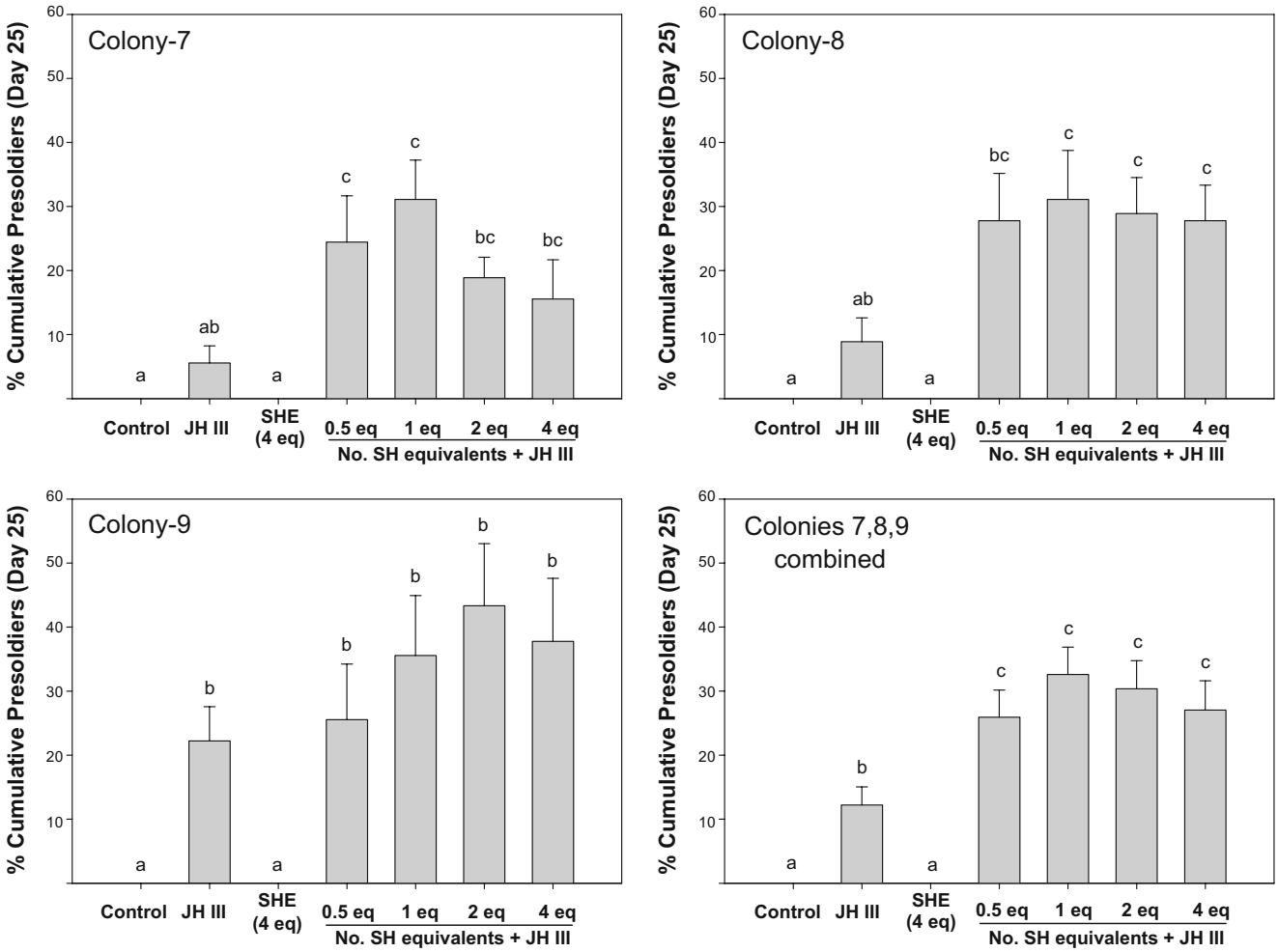

Fig. 1 Percentage formation of presoldier *R. flavipes* in various treatments of workers exposed to soldier head extract (*SHE*) dosages [head equivalents (*eq*)], juvenile hormone III (*JH III*), or control (acetone) for 25 days. Each treatment was replicated six times on three different colonies (7, 8, and 9). The *graphs* for colonies 7, 8, and 9 show cumulative average ± standard error presoldier induction through assay day 25 for each of the separate colonies. The *graph at the bottom right* shows cumulative average ± standard error presoldier induction for the combined colony responses. *Letters* represent significant differences at $P<0.05$

0.002). Although detectable in worker extracts, the respective amounts of the two chemicals was substantially less than in SHE (Fig. 2). Pine wood extracts (Fig. 2) did not have detectable amounts of the two chemicals or of related chemicals, supporting *de novo* production of cadinene and cadinenal.

Cadinene and Previously Identified Soldier Chemicals Enhance JH-Induced Presoldier Differentiation Twelve previously identified soldier head chemicals were tested for ability to induce presoldier formation in dish assays. All of these, except nootkatone and nerolidol, when tested in combination with JH III, caused significant increases in presoldier differentiation relative to JH III alone. When tested without JH III, none caused presoldier differentiation (df=26,63, F=14.46, $P<0.001$) (Fig. 3). Similar to previous assays, no presoldiers were observed in acetone controls, but high levels of presoldier induction (~80%) were observed in SHE+JH III treatments. Treatments of JH III alone induced significantly lower presoldier levels (~20%), comparable to results of preceding experiments (see above).

Discussion

In previous research, termite soldier-produced chemicals have been investigated mostly as a taxonomic tool for species identification (Zalkow et al. 1981; Prestwich 1983; Bagnères et al. 1990; Nelson et al. 2001; Quintana et al. 2003; Nelson et al. 2008). Such research has identified a number of compounds in soldier secretions, but little consideration has been given to roles of these chemicals

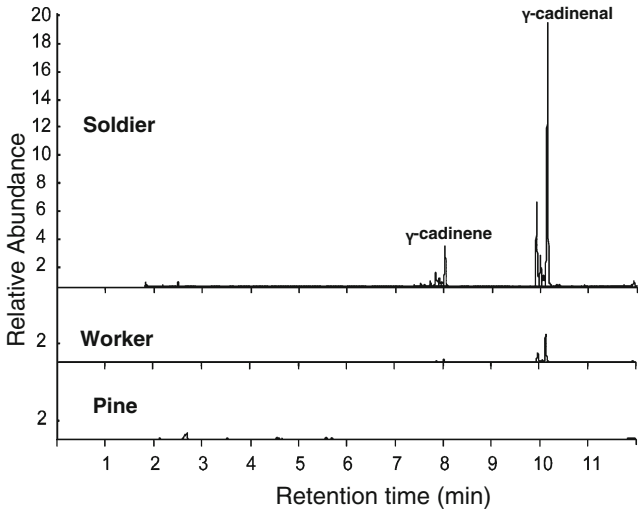

Fig. 2 Gas chromatograms (flame ionization detection) of acetone extracts prepared from heads of 30 soldier (*top*) and worker (*middle*) *R. flavipes*, as well as 1.26 g of seasoned pinewood (*bottom*). Pinewood was seasoned and identical to that used to feed termite colonies

in caste differentiation. The study presented here confirms the effects of *R. flavipes* SHE on JH-induced presoldier differentiation. Results from multiple bioassays on different colonies at different times of the year indicated that SHE synergistically increased worker to soldier morphogenesis when applied in combination with JH III. These findings support the idea that the soldier caste, in addition to playing a defensive role, also plays a part in caste regulation within termite society (Henderson 1998).

Our study also supports previous research showing that ectopic JH III treatments cause some workers to molt into presoldiers (and onto soldiers; Scharf et al. 2003, 2005, 2007; Zhou et al. 2006a, b). The JH III mediated worker-to-soldier molt is an atypical example of a JH III response when compared to other insect groups. In most insects, JH causes insects to remain as immature forms during a molt, while the absence of JH causes the insect to molt into an adult form. Thus, termites have apparently co-opted JH for a different function than other insect groups.

The combination of SHE with ectopic JH III treatments synergistically enhanced presoldier development relative to JH III alone, while SHE by itself caused no presoldier induction. This suggests that SHE probably does not contain significant quantities of JH. Preliminary thin layer chromatographic and GC-MS separations of JH III and SHE showed no common components (MRT unpublished), supporting the absence of JH III in SHE. Therefore, in our assays, we conclude that chemicals from soldier heads modulate the termite response to ectopically applied JH III, thereby enhancing JH III activity. We hypothesize that, endogenously, the synergistic effect of these SHE terpenes is manifest only in individuals with elevated JH titers.

The results from this study are in contrast with past reports that concluded that soldiers and extracts from soldiers inhibit presoldier formation (Lefeuve and Bordereau 1984; Okot-Kotber et al. 1991; Korb et al. 2003). There are several differences between the current and past research that could at least partially explain these discrepancies. First, Lefeuve and Bordereau (1984) exposed groups of 200 workers of the higher termite *N. lujae* to one of three treatments, specifically, nothing, live soldiers, or SHE extracted in DCM. Differences between this and our study include extraction solvent, termite species, and group size. Korb et al. (2003) tested the effect of precocene II, an allatectomizing agent, and SHE extracted in DCM on whole colonies of the lower termite, *C. secundus*. Differences between our study and that of Korb et al. (2003) include solvent, termite species, and treatment size. Korb et al. also did not test precocene in combination with natural JH or SHE. Okot-Kotber et al. (1991) tested combinations of methoprene and SHE extracted in DCM on *R. flavipes* in a dish assay, similar to ours, and found that the combination resulted in less presoldier formation than treatments of methoprene alone. In unreported work, we found no difference between SHE extracted in DCM or acetone (Table S1 and Fig. S1, supplementary material), eliminating the effect of solvent. However, we used JH III in our study while Okot-Kotber et al. (1991) used the JH analog methoprene. Other factors that may explain some of the differences between our study and the preceding ones may be colony conditions at the time of testing and the time of year at which testing was performed, e.g., responses to SHE and JH may vary among termite colonies as well as within a colony over a year according to season.

While our results suggest components of *Reticulitermes* SHE function as primer pheromones, soldier secretions of other termite species have bona fide defensive functions. For example, *Coptotermes* soldiers produce latex that defends against predators (Prestwich 1983, 1984; Abe et al. 2000). Also, the classic example of termite soldier defensive chemicals comes from the higher Nasute termites, which lack mandibles and defend the colony by expelling materials from their frontal gland reservoir onto their enemies (Miura and Matsumoto 2000; Hojo et al. 2005, 2007). Our proposed primer pheromone function for *Reticulitermes* head chemicals is supported by a study by Zalkow et al. (1981) who assayed a number of *R. flavipes* and *Reticulitermes virginicus* soldier head chemicals against the native fire ant, *Solenopsis geminata*. Their results indicated the ants had not been sprayed with an irritant or toxicant and that the soldier head chemicals have nondefensive functions.

No evidence was obtained in the present study to suggest that the chemicals are expelled from soldiers. One

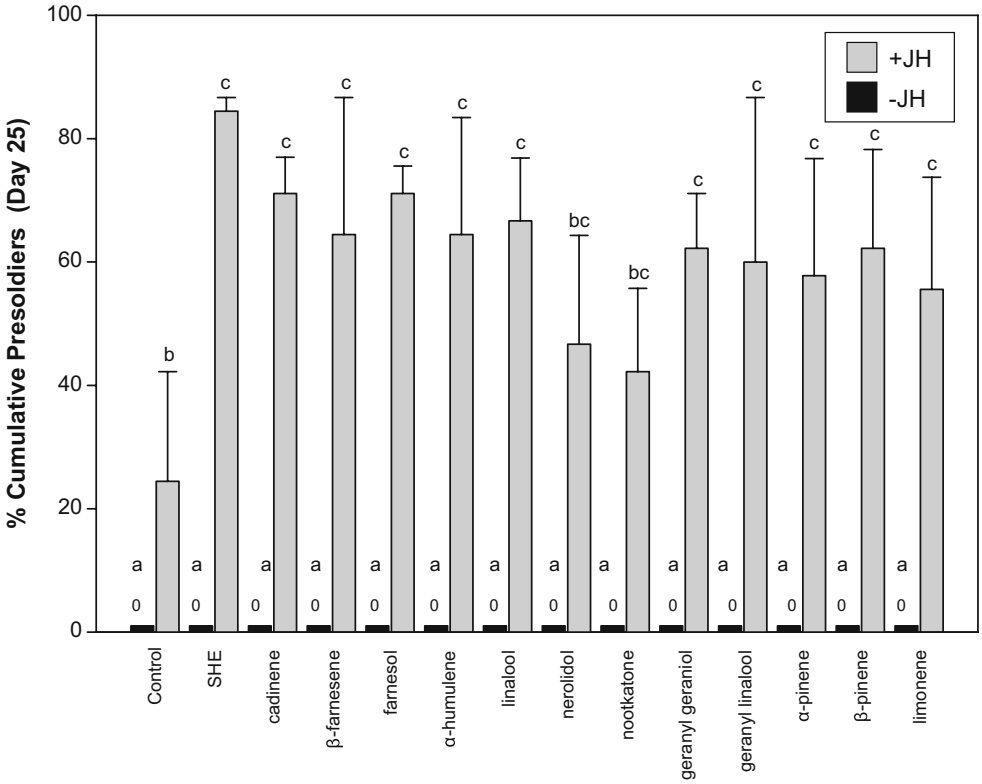

Fig. 3 Twelve previously identified soldier chemicals were tested for their ability to induce presoldier differentiation, alone and in combination with juvenile hormone III (at 150 μg), along with a control (300 μl acetone), and soldier head extract (at four head equivalents). All soldier head chemicals were tested at 50 μg/dish. Each treatment was replicated three times. The *graph* shows cumulative average ± standard error presoldier induction through assay day 25. *Letters* represent significant differences at $P<0.05$

explanation for the soldiers having a large amount of putative primer pheromone in their heads is to serve as a recruiting mechanism after an individual soldier is killed. For example, if a soldier is killed when defending the colony, the chemicals acquired while disposing of the body may signal nestmate workers to differentiate into soldiers. Since workers also contained small amounts of cadinene and cadinenal, another possibility is that soldiers may absorb and sequester these compounds away from workers thus suppressing worker differentiation. For example, live soldiers suppress worker JH titers and inhibit presoldier formation (Park and Raina 2004; Mao et al. 2005). Future research efforts will test hypotheses relating to impacts of live and dead soldiers in nestmate differentiation and terpene sequestration.

Of the soldier head terpenes identified in previous research, all but two significantly enhanced JH-induced presoldier formation when combined with JH III at a ratio of 1:2 (terpene/JH). When applied at the same concentration without JH III, however, none of the terpenes induced presoldier formation. This suggests that *Reticulitermes* have the ability to utilize an array of terpenes as cues to trigger soldier caste differentiation, provided that endogenous JH titers are above critical thresholds. Future research should determine what structural features of the terpenes are necessary for activity.

The regulation of termite caste differentiation is important in maintaining social structure and function, and therefore, the disruption of termite caste differentiation/homeostasis may be able to be used as a control method. By using the termite's own chemistry (i.e., soldier-derived terpenes), it may be possible to develop a specific termiticide that causes a large proportion of worker termites to molt into soldiers. Because soldier termites cannot feed themselves, this would likely cause the termite colony to starve or at least have a severe effect on the colony. For example, in our study (data not shown), mortality was the greatest in replicates in which a high proportion of worker termites molted into presoldiers.

In summary, the findings presented here verify a role beyond defense for the soldier caste within termite societies, as initially proposed by Henderson (1998). These results indicate that non-JH terpenes from termite soldier castes can influence caste polyphenism in nestmates. The results help identify part of the complex chemical communication system that termites utilize to maintain a balanced social environment.

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Factors Influencing Responses to Alarm Pheromone by Larvae of Invasive Cane Toads, *Bufo marinus*

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Abstract If pheromonal communication systems of invasive species differ from those of native biota, it may be possible to control the invader by exploiting that difference. When injured, the larvae of cane toads, *Bufo marinus*, an invasive species of major concern in tropical Australia, produce species-specific chemical cues that alert conspecific tadpoles to danger. Repeated exposure to the alarm chemical reduces tadpole survival rates and body sizes at metamorphosis and, thus, could help control toad populations. To evaluate the feasibility of this approach, we need to know how the intensity of toad tadpole response to the alarm chemical is affected by factors such as water temperature, time of day, larval stage and feeding history, geographic origin of the tadpoles, and habituation. Information on these topics may enable us to optimize deployment, so that tadpoles encounter pheromone at the times and places that confer maximum effect. In our studies, tadpole density, nutritional state, larval stage, and geographic origin had little effect on the intensity of the alarm response, but tadpoles reacted most strongly in higher water temperatures and during daylight hours. Repeated, once-daily exposure to pheromone did not induce habituation, but repeated exposure at 15-min intervals did not elicit further responses after 2 h total exposure. The insensitivity of response to most factors tested means that the effectiveness of the pheromone as a control agent should be relatively robust.

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Introduction

The frequency with which species are being translocated outside their natural range is increasing rapidly due to anthropogenic activities. Thus, there is need of new ways to mitigate the ecological impacts of such taxa (Newsome and Noble 1986; Hengeveld 1989; Parker et al. 1999). One of the primary criteria for any control method is that it targets the invader, without affecting native biota. Such specificity may involve any distinctive trait of the invader, but phylogenetically conservative communication systems offer special promise (Beroza and Knipling 1972; Dalesman et al. 2007). Extensive work with sex pheromones in insects has confirmed the value of this method (e.g., Pelozuelo and Frerot 2006). Vertebrates have attracted less attention in such studies, but may be suited to a similar approach. For example, larvae of many anuran species have well-developed pheromonal communication systems (Summey and Mathis 1998; Spieler and Linsenmair 1999; Rajchard 2006), and Wassersug (1997) suggested that we may be able to exploit such systems to fight invasive species of anurans.

One invasive amphibian of major concern worldwide is the cane toad, *Bufo marinus* (allocated to the genera *Chaunus* or *Rhinella* by some authors—Frost et al. 2006; Pramuk 2006), a large and highly toxic species from Central and South America (Lever 2001). Introduced to more than 30 countries worldwide in attempts to control agricultural pests, toads are now widely recognized as a major ecological problem in many of those areas. For example, the toads introduced to northeastern Australia in

1935 have since spread over tropical areas of that continent, causing extensive mortality (through poisoning) of native frog-eating predators (Burnett 1997; Crossland and Alford 1998; Phillips et al. 2003). Despite massive expenditure, current approaches to cane toad control have failed to curtail the rate of toad invasion (Peacock 2007). New approaches are needed.

Recent work has identified one such possibility. When injured, cane toad larvae produce species-specific chemicals that alert conspecific tadpoles to the proximity of danger (Hagman and Shine 2008a). Tadpoles of native frog species rarely react to this chemical cue (Hagman and Shine 2008b). Importantly, repeated exposure to the pheromone reduces not only toad tadpole survival but also body size at metamorphosis, potentially rendering the newly transformed toad more vulnerable to other mortality sources (Hagman et al. 2009). In combination, these results suggest that applying the chemical cue to natural waterbodies may reduce toad recruitment without collateral damage to native anurans (Hagman and Shine 2008b).

Despite these encouraging findings, many difficulties with practical implementation remain, with extrapolation of laboratory results to the field, problematic. One fundamental question involves the degree to which the intensity of tadpole responses to the alarm chemical depends upon external and internal factors. If the response is seen only over a limited range of external conditions (e.g., water temperatures, times of day, levels of food availability), demographic traits (e.g., population densities), or tadpole characteristics (e.g., geographic location of the source population, stage of larval development), then it may be difficult to deploy the pheromone effectively in the field. Similarly, if tadpoles rapidly habituate to the stimulus, long-term exposure may have little effect on their viability.

Ultimately, empirical evaluation of tadpole responses to such factors should be conducted in the field, and should focus on attributes (such as tadpole survivorship and body sizes at metamorphosis) tightly linked to toad population viability. In the first instance, however, it is easier logistically to conduct such an evaluation in the laboratory and to focus on the behavioral response (avoidance) elicited by the chemical cue. Although less directly linked to toad control, behavioral responses are easier to measure and seem to be part of the causal chain by which alarm chemicals reduce tadpole viability. This paper describes a series of experimental studies designed to evaluate factors that may influence tadpole responses to the alarm pheromone. The work is of interest in more general terms because the proximate determinants of anuran larval responses to chemical cues that predict predator presence are poorly known (Fraker 2008a, b).

Methods and Materials

Study Specimens and Husbandry Adult cane toads collected from populations in Queensland and the Northern Territory provided our breeding stock. Animals were kept in outdoor cages at our facilities on the Adelaide River floodplain in the Northern Territory, Australia. We induced spawning by subcutaneous injections of 0.5–0.75 ml of leuprorelin acetate (Lucrin, Abbott Australasia) diluted in amphibian Ringers' solution to a concentration of 0.25 mg/ml. After eggs hatched, tadpoles were kept in sibling groups of a few hundred individuals in plastic tubs containing 700 l of water (de-chlorinated well water, as was all water used in the experiments described below). We fed tadpoles with boiled lettuce twice a week and changed their water weekly. All tadpoles were raised to stage 21–23 (Gosner 1960) before use, except when stated otherwise.

Experimental Equipment and Procedures We prepared the stimulus by macerating 0.2 g of cane toad tadpoles (rapid crushing ensured that they were killed instantly) in 50 ml of water; this solution was diluted to a volume of 1 l (in water) and then filtered through filter paper. To score tadpole responses, we stained the stimulus green (with food dye: 421067, Queen Fine Foods, Alderley, Qld.), so that we could see when a tadpole encountered it; pilot studies (and controls run simultaneously with treatments) showed that the dye itself did not induce any responses by tadpoles. For controls, we used de-chlorinated and stained well water; these were run at the same time as each experimental group in adjacent containers (randomly allocated). Unless stated otherwise, we used 50 naïve tadpoles per trial. These tadpoles were placed in plastic containers ($0.5 \times 0.4 \times 0.3$ m) with 5 l of water. For a trial, we dispensed 5 ml of freshly prepared stimulus from a syringe along one side of the experimental arena and recorded the behavior of the first 20 tadpoles that encountered the stimulus. In earlier work, we reported that cane toad larvae flee from extracts of crushed conspecifics (Hagman and Shine 2008a, b). We scored repulsion by observing changes to the tadpole's direction of movement when it encountered the stimulus. We scored a tadpole as unaffected if it swam through the stimulus without an overt change in behavior and as repulsed if it abruptly changed its direction in a way that took it away from the stimulus. All procedures were approved by the University of Sydney Animal Ethics Committee.

Does Nutritional Status Influence Tadpole Responsiveness to These Chemical Cues? We compared the numbers of tadpoles repulsed by the stimulus as a function of the time since last feeding. One group was fed the same day as the experiment, whereas food was withheld from the other group for 3 days prior to testing. We used two clutches

from the Fogg Dam population, and our comparisons were between siblings (total of 50 trials per treatment).

Does Water Temperature Influence Tadpole Responsiveness to These Chemical Cues? We scored repulsion to cues from crushed conspecifics at three different water temperatures: 10°C, 21°C, and 31°C. Tadpoles originated from adult toads collected around the towns of Cairns, Normanton (Queensland), Fogg Dam, and Timber Creek (Northern Territory).

Does Time of Day Influence Tadpole Responsiveness to These Chemical Cues? Using tadpoles from the same clutches, as in the previous paragraph, we tested whether the tadpole responses varied throughout the day. We exposed tadpoles to cues from crushed conspecifics every third hour from 0600 to 0300 hours. To clarify the direct influence of illumination levels (versus endogenous circadian periodicity in response), we also conducted the experiment in a darkened room at noon and a brightly illuminated room (using fluorescent lighting) at midnight. Illumination levels averaged 90 lx with the lights on and were undetectably low with the lights switched off (based on a Minolta flash meter).

Does Responsiveness to Chemical Cues from Injured Conspecifics Vary With Ontogeny? We scored repulsion in tadpoles at developmental stages from 20 to 45 (Gosner 1960), in progeny from eight clutches spawned by adult toads from four populations (i.e., two clutches from each population). Two of these populations were from Queensland (Cairns and Normanton), and the other two (Timber Creek and Fogg Dam) were from the Northern Territory.

Does Tadpole Density Influence Larval Responsiveness to These Chemical Cues? We manipulated the number of tadpoles in our experimental arenas to 20, 40, 60, 80, or 100 tadpoles per container and scored repulsion as described previously. For this experiment, we used tadpoles from two clutches of the Fogg Dam population.

Do Cane Toad Larvae Become Habituated to These Chemical Cues? To explore habituation, we conducted two sets of repeated repulsion trials. In one, tadpoles were exposed daily to alarm chemical cues for 15 days in succession. In the other, we exposed a group of tadpoles to alarm chemical cues at 15-min intervals over 5 h. For both of these trials, we used tadpoles from the Cairns and Timber Creek populations.

Results

Does Nutritional Status Influence Tadpole Responsiveness to These Chemical Cues? We never observed repulsion to the control treatment (water with food dye) but both food-deprived and recently fed tadpoles showed high levels of response to the treatment (mean repulsion observed=18.63/20 food-deprived tadpoles, and 16.8/20 recently fed tadpoles). Clutch means were virtually identical ($P>0.99$), so we deleted this factor from future analyses. Two-factor analysis of variance (ANOVA) with pheromone treatment and feeding history as factors revealed a highly significant interaction term ($F_{1,92}=14.32$, $P<0.001$) because the effect of the pheromone on repulsion was higher in food-deprived, than in recently fed, tadpoles. However, the magnitude of the disparity was relatively low, as noted above.

Does Water Temperature Influence Tadpole Responsiveness to These Chemical Cues? We analyzed these data with analysis of covariance (ANCOVA; clutch identity and treatment as factors, temperature as a covariate, number of repulsed tadpoles as the dependent variable). As before, all clutch effects were nonsignificant (all main effects and interactions had $P>0.82$). Repulsion was never observed in controls, but was frequent in tadpoles exposed to chemical cues from crushed conspecifics, especially at high temperatures (Fig. 1a). The interaction between temperature and pheromone effect was significant ($F_{1,272}=867.57$, $P<0.001$) because tadpoles never exhibited repulsion at 10°C, rarely did at 21°C, but almost always did so at 31°C (Fig. 1a).

Does Time of Day Influence Tadpole Responsiveness to These Chemical Cues? We analyzed these data by using repeated-measures ANOVA, with clutch identity and exposure to the alarm pheromone as the factors, time of day as the repeated measure, and number of tadpoles (out of 20) showing repulsion as the dependent variable. Clutch effects were nonsignificant (main effects and interactions, all $P>0.075$). A highly significant interaction between treatment and time of day was apparent ($F_{7,616}=160.42$, $P<0.001$), reflecting a trend for the pheromone to induce stronger repulsion if administered during the morning (highest at 0900 and 1200 hours) than in the afternoon or at night (lowest at 2400 and 0300 hours, see Fig. 1b).

To determine whether this diel variation was induced directly by ambient illumination levels or an endogenous circadian periodicity, we repeated the experiment in a darkened room at noon and an illuminated room at midnight. Repulsion was more common in the darkened room at noon (mean=16.8/20 tadpoles repulsed) than in the illuminated room at midnight (mean=4.6/20 tadpoles repulsed; interaction pheromone treatment×illumination treatment, $F_{1,88}=306.97$, $P<0.001$). Hence, endogenous periodicity had more effect than ambient illumination on the level of tadpole response.

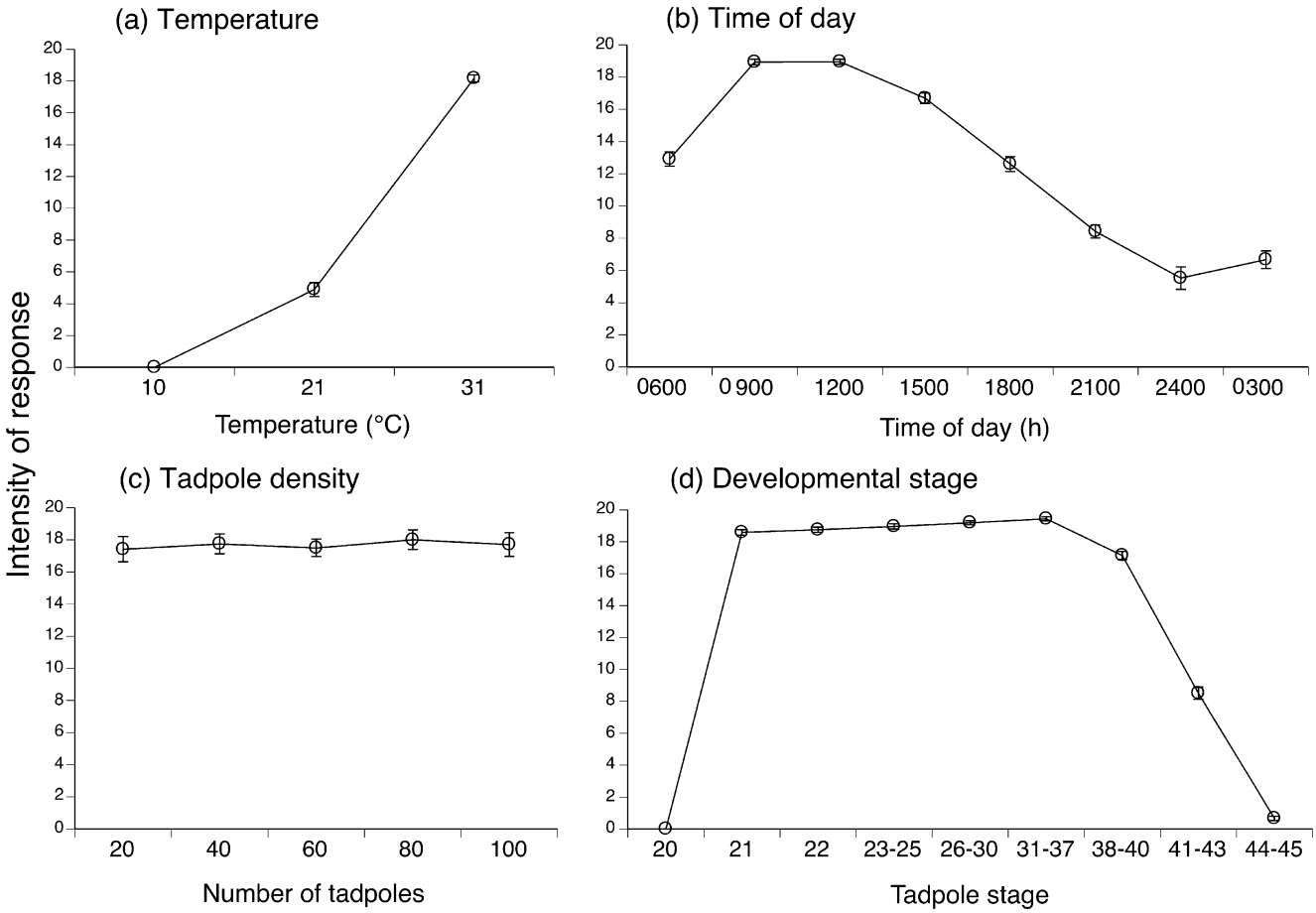

Fig. 1 Effects of experimental conditions on the mean number of cane toad, *Bufo marinus*, tadpoles (out of 20 per trial) repulsed from chemical cues from crushed conspecific tadpoles: **a** water temperature, **b** time of day, **c** tadpole density, **d** tadpole developmental stage. Controls (water without crushed-tadpole cues) were run simultaneously, but never elicited repulsion and are not shown. SEs of means are given

Does Population Density Influence Tadpole Responsiveness to These Chemical Cues? We analyzed these data with ANCOVA (clutch identity and treatment as factors, number of tadpoles per container as the covariate, number of repulsed tadpoles as the dependent variable). Exposure to alarm pheromones increased the incidence of repulsion (main effect, $F_{1,184}=496.72$, $P<0.001$), but no other factors (clutch, density, interactions) influenced repulsion rates (all $P>0.56$). Thus, over the fourfold range of densities used (20 to 100 tadpoles per container), density did not affect the tadpoles' responses to the chemical cue (Fig. 1c).

Does Responsiveness to Chemical Cues from Injured Conspecifics Vary With Ontogeny? Although we used different tadpoles at each developmental stage, we analyzed the resulting data by using repeated-measures ANOVA, with clutch identity and exposure to the alarm pheromone as the factors, developmental stage as the repeated measure, and number of tadpoles (out of 20) showing repulsion as the dependent variable. As before, clutch identity did not explain significant variance (main effect and all interactions, $P>0.12$). A significant interaction was apparent between developmental stage and treatment ($F_{8,704}=1811.34$, $P<0.001$), reflecting nonresponse in the earliest stage, high and consistent responses in tadpoles of Gosner stages 21 to 40 (i.e., most of development), and a diminution of response immediately prior to metamorphosis (stages 41 to 45: see Fig. 1d).

Do Cane Toad Larvae Become Habituated to These Chemical Cues? We analyzed these data by using repeated-measures ANOVA (clutch identity and exposure to the alarm pheromone as factors, successive exposure periods as the repeated measure, and number of tadpoles showing repulsion as the dependent variable). We looked at habituation both over a long timescale (exposure every day for 15 days, comprising most of the total larval period) and a much shorter timescale (every 15 min for 5 h).

In long timescale trials, tadpoles from a Timber Creek (NT) clutch exhibited higher repulsion levels than did a clutch from Cairns (Qld: treatment×population, $F_{1,44}=5.09$, $P<0.03$) throughout the study (interaction time×clutch, $F_{14,616}=0.95$, $P=0.51$). The mean level of repulsion also

changed over time (treatment×day no., $F_{14,616}=2.62$, $P<0.001$). However, the effect was relatively minor and perhaps not biologically significant, with mean response levels per day ranging only from 17.7 to 19.2 repulsions out of 20 (Fig. 2a).

In short timescale trials, response levels were high for the first 2 h, then declined precipitously (time×treatment, $F_{20,880}=1044.25$, $P<0.001$; Fig. 2b). As in the longer-term trials, the Timber Creek tadpoles were more responsive than their Cairns counterparts (treatment×population, $F_{1,44}=4.98$, $P<0.035$) throughout the study (interaction time×clutch, $F_{20,880}=1.31$, $P=0.17$).

Discussion

As in our earlier studies (Hagman and Shine 2008a, b), exposure to chemical cues from injured conspecifics induced a strong avoidance response in cane toad tadpoles, whereas control treatments had no effect. Sensitivity of the pheromone-avoidance behavior to our experimental treatments ranged from minimal (for tadpole density) to acute (water temperature and time of day). Clutch effects (and thus, population-of-origin effects) were minor and generally nonsignificant. Other variables we tested influenced avoidance levels only over a limited range of conditions; for example, tadpoles failed to respond to chemical cues very early and very late in development, but the response was consistent throughout the middle phase of larval life. Although nutritional status had a statistically significant effect (recently fed tadpoles were less responsive), the effect size may be too small to be important biologically. Habituation to daily pheromone exposure was minor or nonexistent, but tadpoles exposed repeatedly at 15-min intervals ceased responding after about 2 h. Below, we examine the likely biological underpinnings of these effects, before considering the implications of our results for deploying alarm pheromone to control cane toad populations.

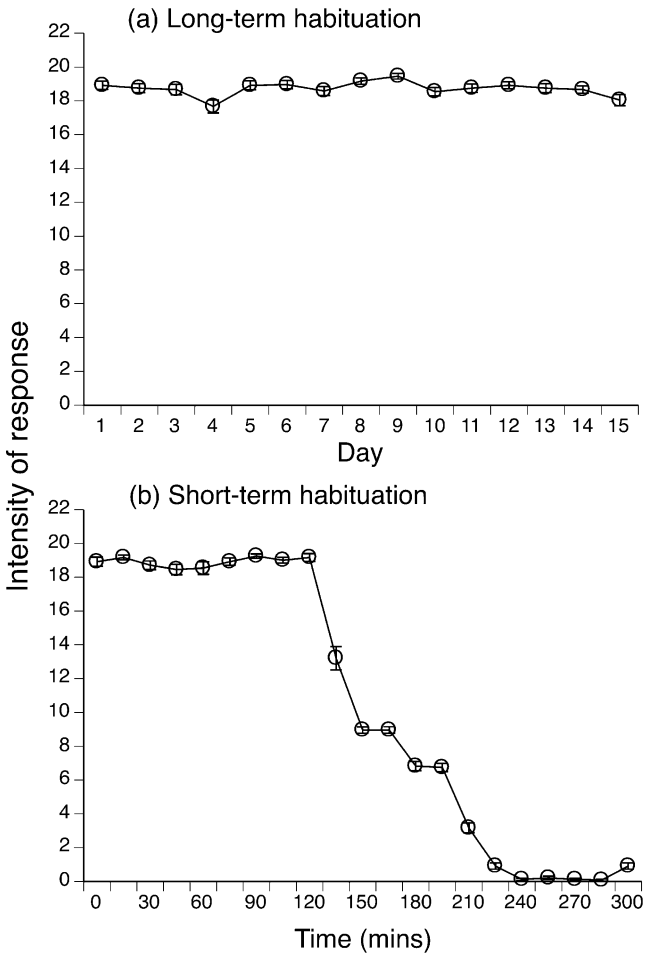

Fig. 2 Effects of **a** long-term (once daily) and short-term (15-min repetitive) exposure on habituation of cane toad, *Bufo marinus*, tadpoles to chemical cues from crushed conspecific tadpoles. The *graphs* show the mean number of tadpoles (out of 20) repulsed by the stimulus per trial. Controls (water without crushed-tadpole cues) were run simultaneously, but never elicited repulsion and are not shown. *Figures* show SEs of means

The results for nutritional state (recently fed tadpoles were less responsive to alarm pheromone) run counter to the pattern reported in previous studies (recently fed tadpoles were more responsive to predator cues: Horat and Semlitsch 1994; Fraker 2008b). Mathematical models predict that the effects of food deprivation on antipredator responses may be sensitive to a wide range of factors (Werner and Anholt 1993). We cannot identify specific reasons for the different patterns seen in our results compared to those of Horat and Semlitsch (1994) and Fraker (2008b). The results may reflect interspecific divergence, a difference in experimental stimuli (conspecific alarm cues vs. predator scent) or a difference in the tadpole behavioral traits elicited by predator cues (flight vs. quiescence). Future work should explore these alternatives.

The strong thermal effect on response intensity (Fig. 1a) cannot be due simply to locomotor ability that increases at higher temperatures: toad tadpoles were capable of swimming around (albeit slowly) even at the lowest temperature that we used in experiments. Hence, the lack of pheromone avoidance at low temperatures may reflect the tadpoles not detecting the chemical cues, or some adaptive matching of response to locomotor ability (i.e., flee only when you are capable of moving rapidly, otherwise ignore the stimulus or remain quiescent). In support of the latter, body temperature has been reported to influence antipredator tactics in many other ectothermic vertebrates (e.g., Huey and Slatkin 1976; Greene 1988; Passek and Gillingham 1997; Cooper 2000; Shine et al. 2000; Langkilde et al. 2003). The lack of the avoidance response in very early stage and very late-stage toad tadpoles (Fig. 1d) is open to the same ambiguous interpretation: it may reflect either limited locomotor ability or an adaptive adjustment of behavior to that ability.

Toad tadpoles reacted more strongly to alarm pheromone during daylight hours than they did at night (Fig. 1b), and experiments under artificial illumination suggest that this is an endogenous circadian rhythm rather than a response to ambient light levels. Recent studies on green frogs (*Rana clamitans*) also have shown that tadpole behavior is more strongly suppressed by predator cues during the day than during the night (Fraker 2008a); the same pattern we documented for cane toads. However, green frog tadpoles forage primarily by night, whereas cane toad tadpoles forage by day (Lever 2001). Thus, the apparent similarity in results of these two studies is surprising. Fraker (2008a) interpreted the strong diurnal response to the lesser importance of diurnal foraging in his study species (and thus, a lowered cost of suppressing feeding activity at that time). This explanation cannot hold for cane toads. Additional work is required to clarify this interspecific difference.

Importantly, the effects of water temperature (Fig. 1a) and time of day (Fig. 1b) will covary because cane toads spawn in shallow ponds exposed to high levels of direct solar radiation (Hagman and Shine 2006; Semeniuk et al. 2007). Water temperatures at 10-cm depth in outdoor ponds near Fogg Dam in October ranged from 22°C in early morning to 38°C by late afternoon; water temperature falls even lower overnight in the middle of the dry season (Hagman and Shine, unpublished data). The effects of diel cycle and water temperature will tend to cancel each other out; for example, water temperatures are low at dawn but tadpole responsiveness is high at this time. The end result of these two influences is likely to be a relative constancy of responsiveness during daylight hours (responsiveness declines during the afternoon, but rising water temperatures will mitigate this effect), but lower responsiveness at night (both because of lowered temperatures and because of the diel cycle in response).

The lack of an effect of tadpole density and the general lack of clutch (and thus, of location-of-origin) effects, are clear-cut null results. The same is true for habituation over the longer timeframe we investigated (Fig. 2a). Such null results are difficult to interpret because there are always more potential reasons why behaviors do not occur than why they do (Gould and Lewontin 1979). It would be interesting to look further at the tadpoles exposed to repeated doses of alarm pheromone at 15-min intervals (Fig. 2b). Why did response levels drop so rapidly after 2 h? This effect could result from tadpole exhaustion, from a shift in response from avoidance to quiescence, or from classical habituation.

Lastly, what do these patterns of response tell us about the feasibility of deploying alarm chemicals in the field to control cane toad populations? Broadly, our results are encouraging, indicating that the pheromone should continue to work effectively day after day throughout the larval phase, regardless of tadpole developmental stage, population density, or feeding rates, and should work over a range of water temperatures typical of breeding ponds in tropical Australia. However, additional research is needed to determine whether or not the alarm pheromone provides a way to reduce cane toad recruitment in nature. For example, we need to know how long the chemical cue persists in nature before it becomes degraded and/or diluted and how long tadpoles continue to respond after being exposed to the cue (Ferrari et al. 2008). Future work could usefully explore the feasibility and effectiveness of deployment of the pheromone in natural water bodies.

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Effects of a *Caenorhabditis elegans* Dauer Pheromone Ascaroside on Physiology and Signal Transduction Pathways

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Abstract Daumone is one of the three purified and artificially synthesized components of the *Caenorhabditis elegans* dauer pheromone. It affects the major signal transduction pathways known to discriminate between developmental arrest at the dauer stage and growth to the adult [the transforming growth factor beta (TGF-β) and *daf-2/IGF1R* pathways], just as natural pheromone extracts do. Transcription of *daf-7*/TGF-β is reduced in pre-dauer larvae, and nuclear localization of the DAF-16/FOXO transcription factor is increased in embryos and L1 larvae exposed to synthetic daumone. However, daumone does not require the cilia in the amphidial neurons to produce these effects nor does it require the Gα protein GPA-3 to induce dauer entry, although GPA-3 is required for dauer induction by natural dauer pheromone extracts. Synthetic daumone has physiological effects that have not been observed with natural pheromone. It is toxic at the concentrations required for bioassay and is lethal to mutants with defective cuticles. The molecular and physiological effects of daumone and natural dauer pheromone are only partially overlapping.

Keywords ASI neurons · ASJ neurons · *Caenorhabditis elegans* · DAF-16 · Dauer · Dauer pheromone · Daumone · Gene expression · Insulin signaling · Physiology · TGF-β · Transcription

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Introduction

Development of the free-living soil nematode *Caenorhabditis elegans* includes an embryonic phase, four larval stages (L1–L4), and the adult. Under conditions unfavorable for reproductive growth, i.e., low food supply, high temperature, and/or high population density, *C. elegans* can enter the dauer stage as an alternative to the L3 (Cassada and Russell 1975). Dauer larvae are a non-feeding diapause state that can survive for months by utilizing fat stores for energy. Once environmental conditions improve, they begin to feed, molt to the L4, and then to the adult (Riddle and Albert 1997).

High population density is signaled by the concentration of a constitutively secreted pheromone (Golden and Riddle 1984a). If the ratio of pheromone to food is high during the L1 and L2 stages, the larvae adjust their metabolism to accumulate fat in preparation for a possibly prolonged period without feeding. An accurate environmental assessment (precise threshold) is highly adaptive. An unnecessary developmental arrest substantially delays reproduction, whereas failure to disperse when necessary negatively impacts progeny survival.

The pheromone was shown to induce dauer formation by affecting several signal transduction pathways. Mutations in some of the genes in these pathways lead to constitutive dauer formation (Daf-c), whereas mutations affecting other pathway components impede entry into dauer, a phenotype called dauer-defective (Daf-d; Riddle and Albert 1997). The genes were placed in pathways based on epistatic relationships [reviewed in Riddle and Albert (1997)]. Two parallel signal transduction pathways, the *daf-7*/transforming growth factor beta (TGF-β) pathway and the *daf-2*/insulin-like growth factor 1 receptor (*IGF1R*) pathway, were defined, converging on the DAF-12 nuclear hormone receptor (Snow and Larsen 2000; Jia et al. 2002).

The dauer pheromone was initially identified as a mixture of hydroxylated short-chain fatty acids or bile salts (Golden and Riddle 1984b). Recently, the four major components of this pheromone were purified from extracts and synthesized (Jeong et al. 2005; Butcher et al. 2007, 2008). Biological activity of these structurally related ascarosides (Fig. 1) was demonstrated by measuring entry into the dauer stage on agar plates in the presence of limited food. One of the pheromone components with the trivial name daumone (Jeong et al. 2005) occurs at about one tenth of the concentration of the two major components (Butcher et al. 2007). We used synthetic daumone to compare its modes of action with natural dauer pheromone extracts with the aim of uncovering the physiological effects and the mechanisms by which daumone induces dauer formation.

Methods and Materials

C. elegans Strains Used Wild-type N2, *mIs7[daf-7* p::*gfp*::*daf-7* 3′ UTR *rol-6(su1006)]* (DR 2021), *zIs356[daf-16* p::*daf-16*::*gfp]* (TJ356) (Henderson and Johnson 2001), *gpa-3 (pk35)*, *daf-3(mgDf90)*, *daf-16(mgDf47)*, *daf-8(m85);daf-3 (e1376)*, *daf-12(m20)*, *daf-14(m77);daf-3(e1376)*, and *dpy-14(e188)* were used. Strains were cultured by using standard techniques (Brenner 1974).

Dauer Induction Assays Assays were performed as described by Jeong et al. (2005). In brief, synthetic daumone (KDR Biotech, Seoul, Korea) was dissolved in 95% ethanol. Plates were prepared by adding daumone to NG agar (Brenner 1974) without peptone to a final concentration of 384 μM. A total volume of 3 ml of NG plus daumone was dispensed in each 50-mm diameter plate.

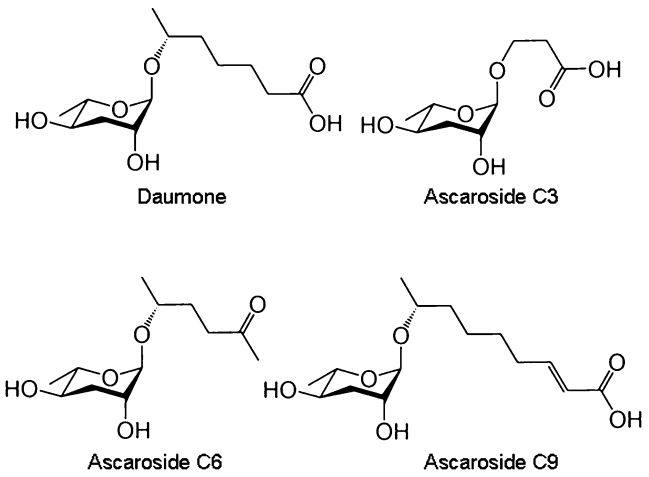

Fig. 1 Structures of the four known components of the dauer pheromone (adapted from Butcher et al. 2008). They are all derivatives of the 3,6-dideoxyhexose ascarilose. Our studies focus on the compound known as daumone

Control plates were prepared by adding 95% ethanol equal to the volume used to deliver daumone to the experimental plates. Heat-killed *Escherichia coli* OP50 (160 μg) were added to the plates and allowed to dry. Five adult worms were placed on each plate and allowed to lay eggs for 5 h before being removed. Plates were then incubated at 25°C for 70–72 h, and dauer formation was scored visually with a stereomicroscope.

Pheromone Plates Pheromone extracts were prepared as described by Golden and Riddle (1984a). Pheromone plates were prepared with pheromone at a final concentration of three times (three times the concentration found in starved liquid culture media) following established protocols (Golden and Riddle 1984b). Control plates were made by adding a volume of M9 buffer equivalent to the volume of pheromone added on the experimental plates.

DAF-16 Nuclear Localization Five *daf-16 p*::*daf-16*::*gfp* (TJ356) adults were placed on daumone or control plates and allowed to lay eggs at 25°C. Nuclear localization was assessed after 14 h by observing green fluorescent protein (GFP) fluorescence at 660× with the aid of a Zeiss Stemi SV 11 Apo stereomicroscope. Embryos or L1 larvae were scored as either having DAF-16 nuclear localization or as intermediate/cytoplasmic localization. *P* values were calculated by using the χ^2 test. Embryonic DAF-16 nuclear localization was similarly assessed on pheromone plates, with the exception that three gravid adults were placed on each plate and allowed to lay 30–50 eggs.

Daumone Toxicity Triton X-100 (Sigma) was used to permeabilize the nematode cuticle. Before seeding plates with OP50, 300 μl of a stock 10% solution of Triton X-100 were added to plates to reach a final concentration of 1% (Rogalski et al. 1990). Control plates were made by adding 300 μl of distilled water instead of detergent. Ten to 15 gravid *dpy-14(e188)* adults were added per plate and incubated at 25°C. Survival was assessed at 16 and 24 h. Statistical analysis was performed with the log-rank test available with the statistical package R.

Results

Daumone Reduces TGF-β Signaling TGF-β signaling promotes reproductive growth, whereas its down-regulation results in dauer formation (Ren et al. 1996). Exposure to dauer pheromone extracts prevents transcription of the TGF-β ligand *daf-7* (Ren et al. 1996), which is solely expressed in the amphid chemosensory neurons ASI. These are a pair of bilaterally symmetrical ciliated neurons that

function to inhibit dauer entry (Bargmann and Horvitz 1991). To test whether daumone possesses similar properties to whole dauer pheromone extracts, we exposed a strain carrying an integrated *daf-7* promoter::GFP fusion (*daf-7* p::*gfp*) to 384 μM daumone. Reporter fluorescence gradually decreased from the L1 to the pre-dauer L2d (Fig. 2c, d) and was completely abrogated in dauer larvae formed in response to daumone.

Although the decreased promoter activity observed in our transgenic larvae recapitulated the effects of whole dauer pheromone extracts (Ren et al. 1996), there was also an obvious difference. About 25% of the larvae treated with daumone expressed the *daf-7* p::*gfp* reporter not only in the ASI neurons but also in another amphid neuron (Fig. 2e). Expression was transient just before dauer formation and was absent in dauer larvae. The cell body of this neuron is just posterior to the midline of the second bulb of the pharynx, a location characteristic of the ciliated amphid neuron ASJ, which is required for exit from the dauer stage (Bargmann and Horvitz 1991). There have been no previous reports of *daf-7* expression in the ASJ neurons.

Daumone Induces DAF-16 Nuclear Localization in Embryos Dauer induction involves down-regulation of *daf-7*/TGF-β and *daf-2/IGF1* signaling. Mutations that disrupt either pathway result in constitutive dauer formation. Insulin/IGF1 signaling inhibits the FOXO transcription factor DAF-16 (Lin et al. 1997). When insulin signaling is reduced, DAF-16 is not phosphorylated by the upstream insulin pathway component AKT-1 and is free to enter the nucleus (Paradis and Ruvkun 1998; Henderson and Johnson 2001) to regulate the transcription of genes involved in dauer formation and adult longevity [reviewed in Jensen et al. (2006)]. If daumone reduces insulin signaling, exposure to daumone should result in the nuclear localization of DAF-16. To test this, we exposed a transgenic line carrying an integrated DAF-16::GFP translational fusion (Henderson and Johnson 2001) to daumone. We scored animals at different developmental stages as having nuclear or cytoplasmic/intermediate DAF-16 localization.

The decision to enter the dauer stage is taken during the L1 and the L2 (Golden and Riddle 1984a), and indeed, nuclear localization appeared to be stronger in daumone-treated L1s (Fig. 3) than in L1s grown on control plates, although this difference did not reach statistical significance (P=0.228). Surprisingly, daumone induced strong DAF-16 nuclear localization in embryos (P<0.001). It did not do so in adults (data not shown). If embryonic DAF-16 nuclear localization is a requirement for dauer formation, it should be elicited by exposure to natural pheromone extracts as well. We treated DAF-16::GFP worms with pheromone extracts at a concentration sufficient to induce 61% dauer formation in N2. However, we observed no difference in embryonic nuclear localization in worms treated with pheromone compared to control (P=0.64).

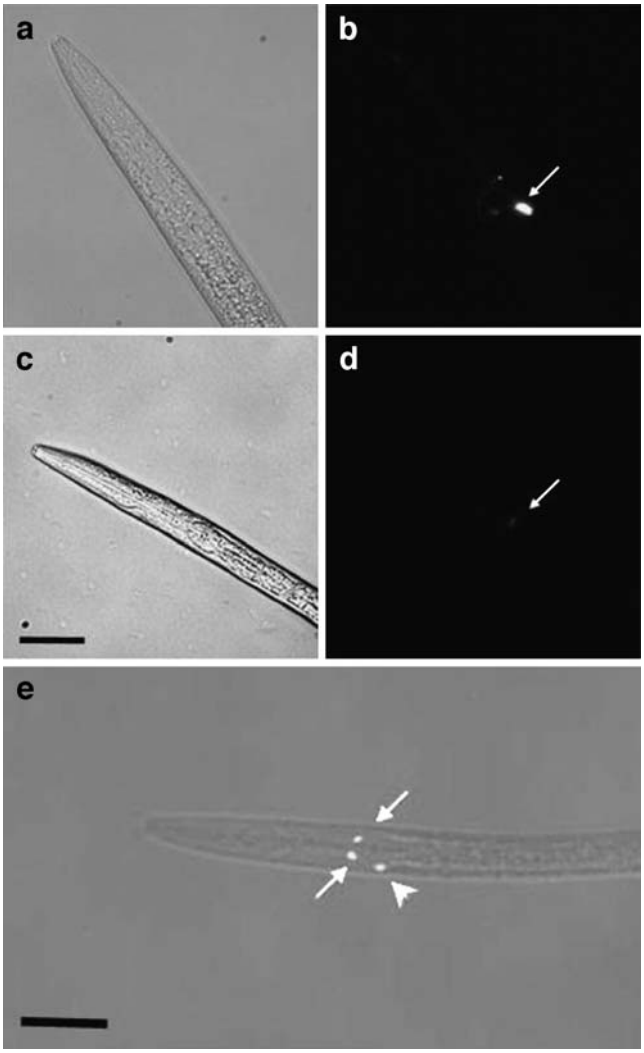

Fig. 2 a–d Daumone decreases expression of *daf-7*, the gene encoding a *Caenorhabditis elegans* TGF-β ligand. Adult hermaphrodites carrying an integrated *daf*-7 p*;;gfp::daf-7* 3′ UTR construct (DR2021) were placed on control or daumone plates and were allowed to lay eggs overnight. After 14-h incubation, larvae were scored for GFP fluorescence with a UV stereomicroscope. **a** DIC and **b** fluorescent image of a control L2 larva. Control larvae showed strong expression of the GFP reporter construct in the ASI neurons. **c** DIC and **d** fluorescent image of a larva hatched in daumone. Larvae exposed to daumone showed little or no GFP reporter expression. **e** Daumone induces expression of *daf-7* in the ASJ neurons. *daf-7* is normally expressed in the ASI neurons (*arrows*). However, ~25% of DR2021 L2d worms treated with daumone expressed *daf-7* in one of the two ASJ neurons (*arrowhead*). The pre-dauer L2d shown is representative of the subpopulation expressing the reporter in ASJ. Scale bars, 50 μm

DAF-16 nuclear localization can be achieved by repression of the insulin pathway or by activation of a stress response pathway parallel to the insulin pathway. This pathway is mediated by the sirtuin SIR-2.1, which binds to DAF-16 and translocates to the nucleus (Henderson and

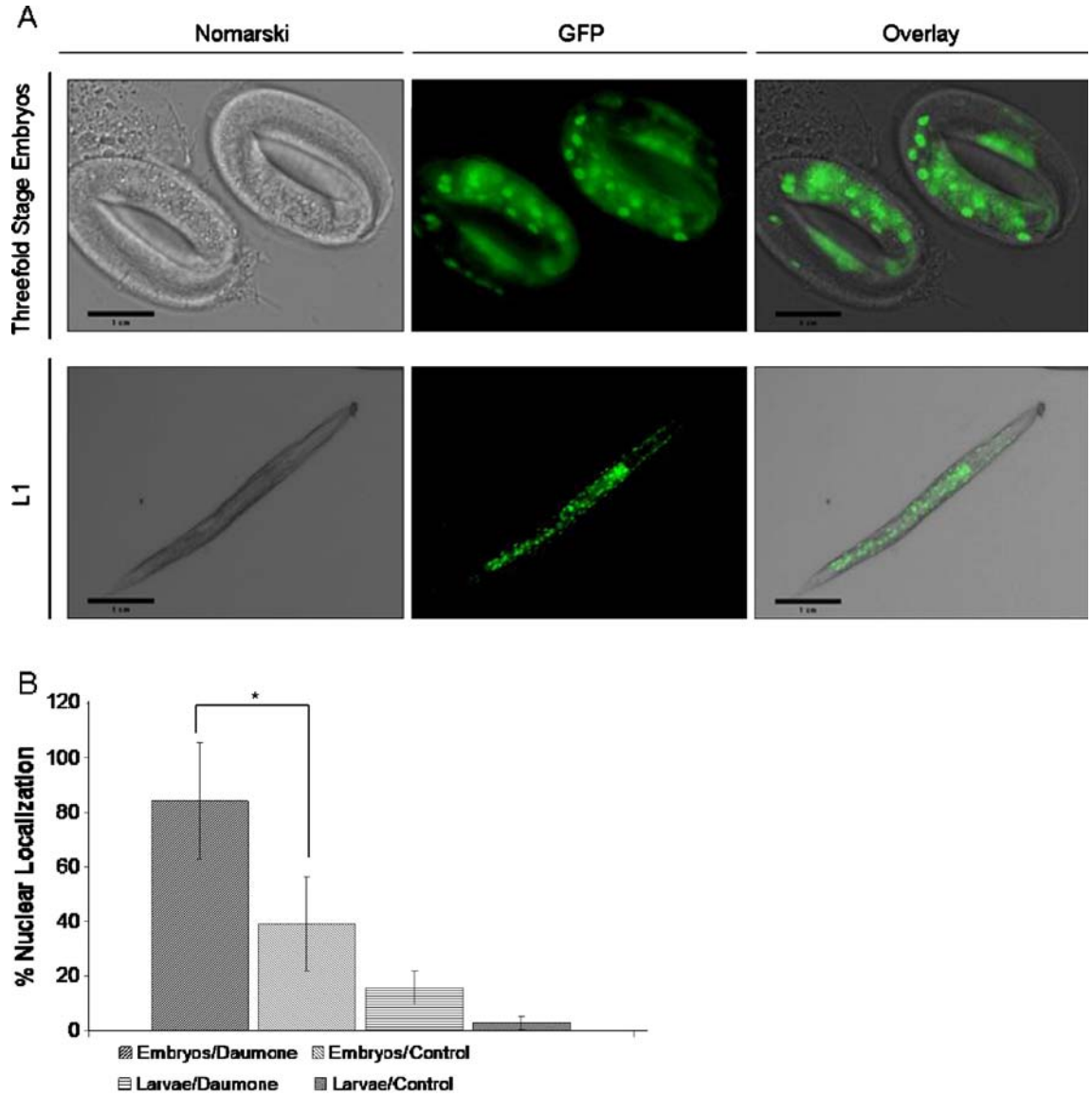

Fig. 3 Daumone induces DAF-16 nuclear localization in embryos. **a** A representative embryo and a representative L1 larva with DAF-16 nuclear localization upon daumone treatment. Strain TJ356 carries an integrated DAF-16::GFP translational fusion construct. **b** Quantification of DAF-16 nuclear localization in embryos and L1s. Animals were described as having nuclear localization if DAF-16 was observed predominantly in the nuclei; otherwise, they were scored as intermediate/cytoplasmic localization. *P* values were calculated with a χ^2 test. *Asterisk* Statistically significant difference. Error bars represent ±standard deviation (SD)

Johnson 2001; Berdichevsky et al. 2006). The DAF-16 nuclear localization in embryos raised the possibility that this was a response to stress (embryos do not enter the dauer stage, and natural pheromone does not induce DAF-16 nuclear localization). The only difference between treatment and control plates was the presence of daumone. Hence, daumone may have toxic effects on *C. elegans* at the concentration used (384 μM; Jeong et al. 2005). Pheromones usually are active in the nanomolar concentration range or a few hundred molecules per square centimeter (Dulac and Torello 2003), much less than the working concentration of daumone required to induce dauer formation in the bioassay (Jeong et al. 2005).

Daumone Toxicity Mutations in the collagen gene *dpy-14* (DumPY) result in a defective cuticle that allows normally excluded substances to leak into mutant worms (Gallo et al. 2006). We used this strain as a sensitized background to perform a daumone resistance assay. To permeabilize the cuticle of these mutants further, we added 1% Triton X-100 to the plates. This concentration of Triton X-100 does not affect animal growth and reproduction (Rogalski et al. 1990). Detergent-treated *dpy-14* adults started dying within 16 h of daumone exposure, as judged by the absence of pharyngeal pumping and lack of response to prodding (Fig. 4). Dpy-14 adults on control plates [where daumone was replaced with 95% ethanol (Jeong et al. 2005)] did not

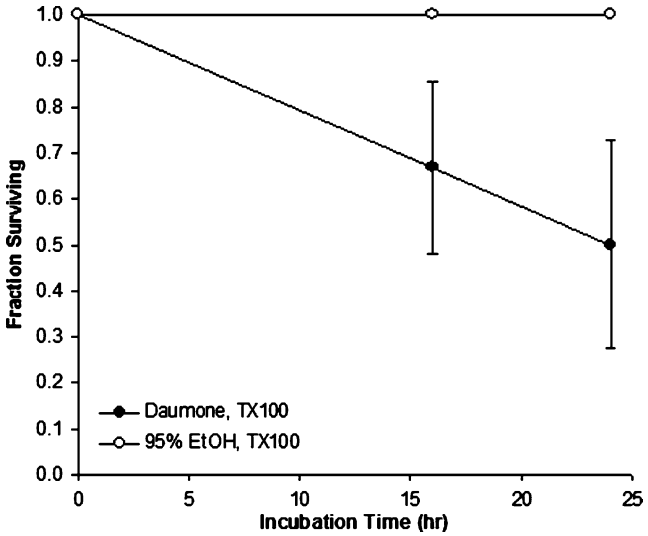

Fig. 4 Daumone is toxic to *dpy-14* mutants with a defective cuticle. This survival assay was performed in duplicate with a total of 18 treated and ten untreated adults. Daumone is lethal to *dpy-14* adults at the concentration used, whereas none of the unexposed mutant adults died. Error bars represent ±standard error of the mean (SE)

die in the first 24 h of daumone treatment. Hence, *dpy-14* adults exposed to daumone had a lower survival rate than controls (P=0.009 by log-rank test). If adults with a Rol-6 (roller) phenotype were placed on daumone plates, they also died or looked sick within a few hours. *rol-6* encodes a cuticular collagen and mutations in this gene result in a cuticle twisted in a right-handed helix (Kramer et al. 1990).

Genes Required for Daumone-Induced Dauer Entry Since the DAF-16 nuclear localization in embryos and L1 larvae could be a response to stress, we wanted to know what pathways might be required for dauer induction with daumone. We employed mutants that down-regulate the *daf-2/IGFR1*-insulin and *daf-7*/TGF-β pathways in dauer induction assays with daumone. The results point to a role for the Smad protein DAF-14 in relaying the daumone signal (Fig. 5). The *C. elegans* TGF-β pathway [reviewed in Riddle and Albert (1997)] includes the DAF-7 ligand, the receptor heterocomplex formed by DAF-1 and DAF-4, and the two receptor Smads (R-Smads) DAF-8 and DAF-14. Activation of these R-Smads results in reproductive growth, whereas mutations that decrease their function result in a Daf-c phenotype. DAF-8 and DAF-14 antagonize the Smad family member DAF-3, which functions with the Sno/Ski family protein, DAF-5, to promote dauer formation. Mutations in DAF-3 and DAF-5 result in a Daf-d phenotype.

The downstream Daf-d mutant *daf-3* suppresses the *daf-8* and *daf-14* Daf-c mutations. Nevertheless, daumone induces dauer formation in such genetic backgrounds (Fig. 5). A failure to respond to daumone indicates that *daf-12* and *daf-14* are required for daumone induction. By contrast, *gpa-3*, *daf-3*, *daf-8*, and *daf-16* are not required. Mutants in *daf*-3 (N=38) or *daf-8; daf-3* double mutants (N=78) responded to 384 μM daumone similar to wild type, forming 50–60% dauer larvae. However, when DAF-14 function was reduced, as in our *daf-14; daf-3* double mutants, dauer formation in response to daumone was severely compromised (5.3%, N=86). This level of dauer formation is actually comparable to that of a *daf-12* (Daf-d) mutant exposed to daumone (6.2% dauer formation, N=103). Our results are, therefore, consistent with genetic data suggesting that the *daf-2/IGF1R* and TGF-β pathways converge on *daf-12* (Riddle and Albert 1997).

One unexpected result was produced by the *gpa-3* (G-protein subunit α) knockout mutant (Fig. 5). GPA-3 is a candidate for relaying the dauer pheromone signal from the pheromone receptors to downstream effectors (Lans et al. 2004), including DAF-11. *gpa-3* and the *daf-11* transmembrane guanylyl cyclase have been shown to interact at the

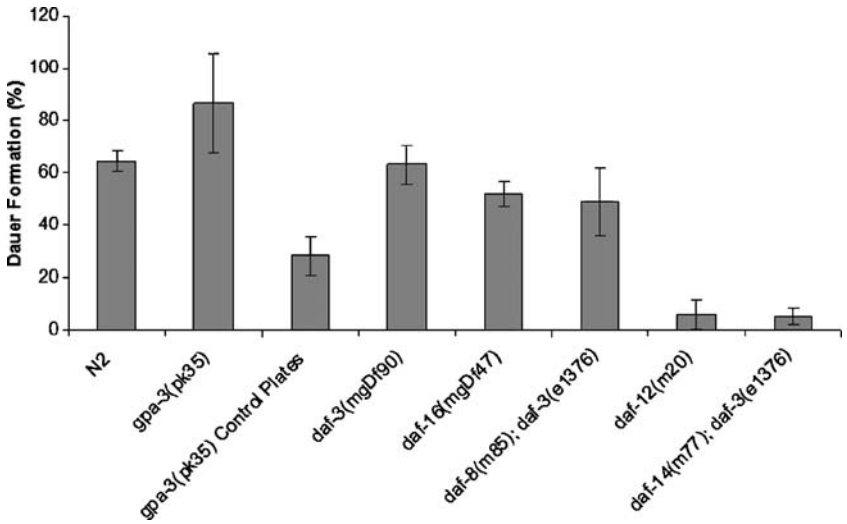

Fig. 5 Response of Daf mutants to daumone. Selected Daf-d mutants in the *daf-2/IGF1R* and TGF-β pathways were used in dauer induction assays with daumone. N2 with daumone treatment and *gpa-3* on daumone assay plates without daumone were used as controls. Bars represent ±standard deviation (SD)

genetic level and have been placed in a pathway both in parallel and partially upstream of the TGF-β pathway (Zwaal et al. 1997; Birnby et al. 2000). *daf-11* mutations result in decreased cyclic guanosine monophosphate (cGMP) levels and constitutive dauer formation (Birnby et al. 2000). cGMP is used as a signaling molecule to modulate many cellular functions through the activation of kinases, other nucleotide cyclases, cyclic nucleotide phosphodiesterases, and cGMP-gated ion channels [reviewed in Goy (1991)].

The *gpa-3(pk35)* knockout strain has a Daf-d phenotype when exposed to pheromone extracts (Zwaal et al. 1997). However, exposure of the Daf-d *gpa-3(pk35)* nulls to daumone resulted in 87% dauer formation (N=60), a significantly higher dauer formation rate than *gpa-3(pk35)* grown on control plates without daumone (28%, N=31). This result suggests that dauer pheromone and daumone induce dauer formation by partially different pathways. The pheromone requires *gpa-3* for dauer induction but daumone does not.

Discussion

This work describes the effects of daumone on *C. elegans* physiology and signal transduction pathways. The optimal concentration for dauer induction assays proposed by Jeong et al. (2005) is about 1,400-fold higher than the concentration of this compound in culture media (Butcher et al., 2007). Butcher et al. (2007) determined the dose–response effect of daumone on dauer formation. From that study and ours, it seems that high concentrations of daumone (384 μM) are required to produce dauer arrest in the absence of the most potent dauer-inducing ascarosides. At this level of exposure, daumone has a lethal effect on animals with permeable cuticles and results in embryonic nuclear localization of DAF-16, suggesting that it may induce dauer formation via a stress response pathway. DAF-16 activity is regulated by insulin signaling via AKT kinases and by a parallel stress response pathway involving SIR-2.1 (Henderson and Johnson 2001; Berdichevsky et al. 2006).

Mutations in *daf-7* result in DAF-16 nuclear localization (Lee et al. 2001), so it is possible that the nuclear localization we observed was due partially to a daumone-induced decrease in *daf-7* gene function. However, DAF-16 nuclear localization in embryos seems to be caused primarily by daumone toxicity. A fluorescent derivative of daumone has been reported to penetrate the egg shell and enter the embryo (Baiga et al. 2008). Our data suggesting that daumone is toxic are in agreement with recent findings of Kim and Paik (2008), who used daumone to induce dauer formation in N2 and to keep them in the dauer stage for increasing lengths of time. They observed that the longer the exposure to daumone, the more severe were the developmental and reproductive defects in post-dauer animals. Such defects were not observed in starvation-induced dauer larvae (Klass and Hirsh 1976).

In spite of its toxic effects, the use of daumone has proven useful for dissecting the functional relationship between the three major ascarosides in the dauer pheromone. Our experiments suggest that daumone does not act on all the canonical pathways for dauer entry. For instance, daumone does not require *gpa-3* to induce dauer entry, although this gene is necessary for dauer induction by natural dauer pheromone extracts (Zwaal et al. 1997). This result suggests that daumone is not sensed by the ciliated neurons, where *gpa-3* is expressed, but instead, it uses an alternative path into the animal. In fact, *daf-10* mutants are Daf-d, but they form 82% dauer larvae at 25°C when exposed to daumone (Jeong et al. 2005). *daf-10* mutants have defective intraflagellar transport and their cilia—rarely formed—are not functional (Qin et al. 2001). *daf-10* does not form dauer larvae when exposed to natural pheromone extracts (Golden and Riddle 1984b). All together, these data suggest that daumone (at the concentration used) does not require the ciliated neurons as does the natural dauer pheromone. Daumone may enter through the cuticle, as this ascaroside is lethal to animals with a permeable cuticle.

Mutations in the FOXO transcription factor *daf-16*, which is the main effector of insulin/IGF1 signaling, do not prevent dauer formation in response to daumone nor do mutations in members of the TGF-β pathway. When *daf-16* is mutated, *daf-3(+)* in the TGF-β pathway may be sufficient for dauer entry in response to daumone. Likewise, when *daf-3* is mutated, *daf-16(+)* may be sufficient to mediate dauer formation. However, DAF-14 is necessary for daumone-mediated dauer entry, suggesting that DAF-14 is required by DAF-16 and DAF-3 to induce dauer formation. When DAF-14 and DAF-3 have reduced function [as in our *daf-14(m77); daf-3(e1376)* double mutant], DAF-16 is not able to induce dauer formation. Interaction between SMAD and FOXO transcription factors has been reported in human cells (Seoane et al. 2004) and was also proposed for DAF-16 and DAF-3 under dauer-inducing conditions in *C. elegans* (Ogg et al. 1997). It is possible that daumone enhances DAF-16/DAF-3 interaction in a DAF-14-dependent manner. The interactions proposed in this paper explain what we observe when animals are treated with daumone and do not necessarily correspond to the pathways normally used for dauer entry.

In summary, daumone, a minor component of the dauer pheromone, needs to be used in synthetic forms at concentrations so high to induce dauer formation that it becomes toxic. It does not fully recapitulate the events that

normally lead to *C. elegans* dauer formation. Our results suggest that dauer formation may be induced partially in response to toxins, in addition to the environmental cues already known (pheromone, food, and high temperatures). The pheromone cue may normally involve the orchestrated action of daumone, the two major ascarosides, and other pheromone components found in the natural dauer pheromone extracts by Butcher et al. (2007) that trigger the complex signaling processes revealed by the genetics. Different ascarosides may activate different sensory receptors, and at physiological concentrations, the behavioral response may result from parallel activities. The chemical composition of the pheromone may contain specific information about environmental status.

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The Chlorophyll Catabolite, Pheophorbide *a*, Confers Predation Resistance in a Larval Tortoise Beetle Shield Defense

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Abstract Larval insect herbivores feeding externally on leaves are vulnerable to numerous and varied enemies. Larvae of the Neotropical herbivore, *Chelymorpha alternans* (Chrysomelidae:Cassidinae), possess shields made of cast skins and feces, which can be aimed and waved at attacking enemies. Prior work with *C. alternans* feeding on *Merremia umbellata* (Convolvulaceae) showed that shields offered protection from generalist predators, and polar compounds were implicated. This study used a ubiquitous ant predator, *Azteca lacrymosa*, in field bioassays to determine the chemical constitution of the defense. We confirmed that intact shields do protect larvae and that methanol-water leaching significantly reduced shield effectiveness. Liquid chromatography-mass spectrometry (LC-MS) of the methanolic shield extract revealed two peaks at 20.18 min and 21.97 min, both with a molecular ion at *m/z* 593.4, and a strong UV absorption around 409 nm, suggesting a porphyrin-type compound. LC-MS analysis of a commercial standard confirmed pheophorbide *a* (Ph*a*) identity. *C. alternans* shields contained more than 100 μg Ph*a* per shield. Shields leached with methanol-water did not deter ants. Methanol-water-leached shields enhanced with 3 μg of Ph*a* were more deterrent than larvae with solvent-leached shields, while those with 5 μg additional Ph*a* provided slightly less deterrence than larvae with intact shields. Solvent-leached shields with 10 μg added Ph*a* were comparable to intact shields, even though the Ph*a* concentration was less than 10% of its natural concentration. Our findings are the first to assign an ecological role for a chlorophyll catabolite as a deterrent in an insect defense.

Keywords Chrysomelidae · Cassidinae · *Azteca* · *Chelymorpha* · porphyrin · *Merremia* · Insect defense

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Introduction

More so than for agile insect adults, flightless, soft-bodied larvae are exposed to numerous natural enemies as they feed openly on leaf surfaces. Consequently, folivorous larvae suffer the highest mortality rates, frequently approaching 90%, among the respective developmental stages (Gross 1993; Hunter 2000; Zalucki et al. 2002). In response, larvae have evolved an astonishing variety of physical barriers, such as setae, spines, and webs, as well as chemical barriers, such as glandular exudates and fore and aft enteric discharges that function to reduce vulnerability to natural enemies (Whitman et al. 1990; Evans and Schmidt 1991; Ruxton et al. 2004 and references therein). Many of these strategies are supplemented by behaviors that enhance their effectiveness (Gross 1993; Stamp and Casey 1993 and references therein). Among the most bizarre of these characters is fecal retention. Although rare among insects generally (Weiss 2006), fecal retention can be found in a restricted number of leaf beetle (Chrysomelidae) and weevil (Curculionidae) lineages with exophytic larvae. Within the chrysomelids, however, fecal retention is

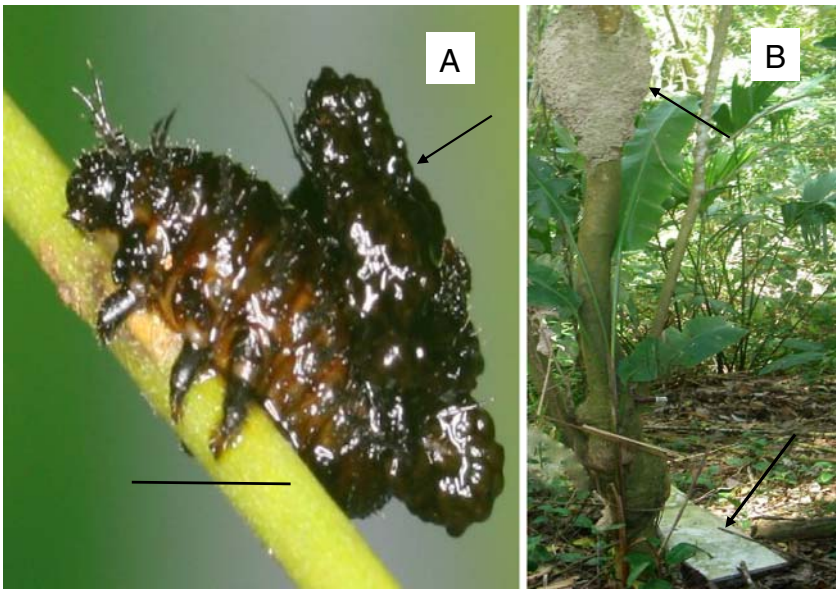

Fig. 1 Larva of **a** *C. alternans* showing the fecal shield (*arrow*) above the larval dorsum (*bar*= 2 mm); **b** the *A. lacrymosa* nest (*upper arrow*) and bioassay arena (*lower arrow*)

common. Nearly 20% of the 35,000 described leaf beetle species have some form of larval fecal retention (Vencl et al. 1999). For example, there are three different approaches to using feces for larval defense in the Chrysomelidae: the unstructured dorsal piles in Criocerinae, the scatoshells in the Camptosomata group, and the elaborate dorsal annexes constructed by the Cassidinae.

Instead of ridding themselves of their digestive wastes, the larvae of tortoise beetles (Cassidinae) have the peculiar habit of voiding feces onto their backs, where it accumulates to form a coating or shield. Shields do not rest passively on the dorsum. They have a solid infrastructure formed by the accumulation of molted skins, which is itself attached to a highly movable, forked caudal process located on the penultimate abdominal segment. Adding to the shield system is a novel, telescoping anus that precisely applies feces to the shield armature (see Vencl et al. 1999; Fig. 1a). Fresh, wet feces may be the most deterrent or repellent part of the elaborate shield structure, and the unusual anal application mechanism maintains the freshest fecal material right at the periphery of the shield where predators are most likely to make initial contact (Vencl, personal observation). Held like parasols above the larvae, shields can be aimed and waved in any direction. Although possibly affording protection against deleterious abiotic factors, tortoise beetle shields have been shown to function as both physical (Eisner et al. 1967; Olmstead and Denno 1993; Eisner and Eisner 2000) and as chemical defenses against natural enemies (Vencl et al. 1999; Müller and Hilker 2004; Gómez et al. 1999; Nogueira-de-Sá and Trigo 2002, 2005).

Larvae of the tortoise beetle, *Chelymorpha alternans*, possess fecal shields that previously have been shown to thwart attacking bugs and ants (Fig. 1a). Although the role of defensive behaviors, such as shield waving and escape, may have contributed to larval survival, the effectiveness of *C. alternans* shields appeared to be due mainly to a chemical component residing in the shield's feces. For example, when larvae lacking recourse to defense behaviors had their shields leached with methanol (MeOH), their capacity to withstand capture by ants was reduced significantly, compared to both larvae with water-leached and larvae with intact shields. This finding indicated that this species relies heavily on MeOH-soluble components to mount an effective defense (Vencl et al. 2005). Although shields in some tortoise beetle species depend on host-derived terpenes, phenolics, alkaloids, or saponins, the shields of other species require fatty acids or phytol to function effectively as defenses (reviewed by Müller and Hilker 2004). However, neither the chemical constitution nor the origin of *C. alternans*' shield chemistry is known.

Here, we investigated how *C. alternans* larvae chemically enhance their remarkable shield structures. We wanted to know what compounds might be responsible for shield effectiveness, whether chemically denuded shields could confer resistance against one of the larva's most important enemies, if larvae were somehow able to sequester repellent or deterrent precursors from their diet, or if they were capable of endogenously synthesizing them. Based on the previous study mentioned above, we focused on the bioactivity of the methanolic extract of *C. alternans* shields, which appeared to be responsible for reduced larval vulnerability to predation.

Materials and Methods

Beetle Collection and Husbandry We collected *C. alternans* larvae from *Merremia umbellata* (Convolvulaceae)

near Gamboa, Colon Province, Republic of Panamá (9°06′ N, 79°41′ W). Larvae were transported to a laboratory at the Smithsonian Tropical Research Institute in Gamboa and maintained at ambient photoperiod and temperature separately in plastic food containers (473 ml) with plastic mesh for aeration and moistened filter paper. Each cup was supplied daily with a fresh, intact *M. umbellata* leaf. Upon reaching the fourth instar, wet and dry weights of larvae and shields (N=34) were taken to the nearest 10^{-4} g. Shields for chemical analysis were harvested and stored without solvent at −32°C. To focus on resistance due to shields alone, we eliminated confounding escape and shield waving behaviors by using freshly killed (by freezing) fourth instar *C. alternans* larvae. Larvae were stored individually in 2 ml glass vials at −2°C for later use in bioassay experiments.

Separation and Identification of Active Compounds in the Shield Extract Liquid chromatography-mass spectrometry (LC-MS) was used to identify the constituents of the methanolic extract. We dissolved 100 mg of shield material in 2 ml MeOH, sonicated it for 5 min, and then centrifuged the sample for 20 min. The supernatant was placed in a gas chromatography (GC) vial and diluted 1:10, of which 10 μl were injected into an Agilent HP 1100 high-performance liquid chromatography (HPLC) equipped with a Lichrosphere RP18, 5 μm column. A water–CH_3CN gradient with a flow rate of 1 ml min^{-1} started at 70:30 CH_3CN/H_2O (*v*/*v*) from 0–30 min to 100% CH_3CN up to 45 min hold at 100% CH_3CN and then 45–45.5 min back to 70:30% CH_3CN/H_2O (*v*/*v*) for 60 min. The sample was analyzed by a Thermo Finnigan LCQ with a positive atmospheric pressure chemical ionization source, in full scan mode with a vaporizer temperature of 450°C, a capillary temperature of 145°C, a sheath gas flow rate of 60 arbitrary units, and an auxiliary gas flow of 15 arbitrary units. The discharge current was 5 eV, the capillary voltage 10 V, and the tube lens offset was 20 V.

Verification of Compound Bioactivity Based on the LC-MS results, a commercial pheophorbide *a* (Ph*a*) standard (Wako 163-11171) was applied to shields to measure repellence or deterrence in field bioassays. One day before a bioassay experiment, larvae were removed from storage and their shields detached by placing fine forceps between the tines of the caudal process and gently lifting the shield away from the larva. Larval bodies were returned to refrigeration at 0°C.

Shields were randomly assigned to either a Ph*a* treatment group, to an intact shield "control" group, or to a solvent control group. The shield groups receiving Ph*a* test solutions were subjected to the following leaching treatment to remove metabolites: 200 ml of MeOH for 45 min with agitation every 5 min, followed by 400 ml of H_2O for 45 min with agitation every 5 min. Shields were dried under a fan for 1 h and then placed individually in 2 ml glass vials. From a 1 mg ml^{-1} methanolic stock solution of pheophorbide *a*, dilutions were made to obtain 1, 3, 5, or 10 μg test dilutions (N=32, 36, 34, and 32 shields, respectively). For example, to apply 1 μg of Ph*a* to a shield, 40 μl of the stock solution was placed in a vial to yield 40 μg, which were then dissolved in 2 ml of MeOH. From this solution, 50 μl (1 μg) were topically applied to each shield. Shields were dried under a fan for 12 h. Each shield was reattached to the caudal process of a larva by using a rapid setting, fumeless, water-insoluble craft glue (DAP®), and allowed to dry 2 h before bioassays began. Shields in the solvent control group (N=41) were leached and dried as per above, and each shield received 50 μl of MeOH. Shields were dried 2 h before bioassays began. Unleached, intact controls (N=32) consisted of only the shield removal and reattachment manipulations described above.

Bioassay Protocol Field bioassays were conducted near Gamboa in September 2007, between 8:30 A.M. and 12:30 P.M. We used a common and aggressively recruiting generalist predator, the ant *Azteca lacrymosa* Forel (Hymenoptera: Formicidae: Dolichoderinae), in the bioassays. Voucher specimens of host, beetle, and ant species are deposited in the herbarium and insect collection of Smithsonian Tropical Research Institute, Panamá.

Bioassay experiments were conducted on a raised, 45×60×2 cm platform that was touching the bole of a tree with an *A. lacrymosa* nest (Fig. 1b). Vines and fallen branches with active ant trails were connected to the platform, and these formed clearly defined avenues across the platform's surface, which varied from four to seven daily. To verify foraging motivation in the ants, the platform was baited with tuna and sugar water 1 h prior to experiments. If baits were removed within the hour, an experiment was conducted with one of the treatments. Each bioassay trial consisted of the presentation of a single shielded larva. With soft forceps, we placed the larva 1 cm from an active foraging trail on the platform. A trial began after the first ant antennated the larva. Each bioassay trial lasted 5 min, or until a larva was captured. A larva was deemed captured when the ants carried it ≥1 cm sideways or backwards, and we recorded this interval as the time from first contact to capture. Individual trials were separated from one another by 3 to 5 min and were conducted along different trails on the platform separated from one another by at least 30 cm. These time delays and variable locations minimized recruitment interactions across assays to insure that different ants were involved in each bioassay trial. Trials of one of the treatment types or of intact larvae were interspersed randomly with trials of the solvent controls.

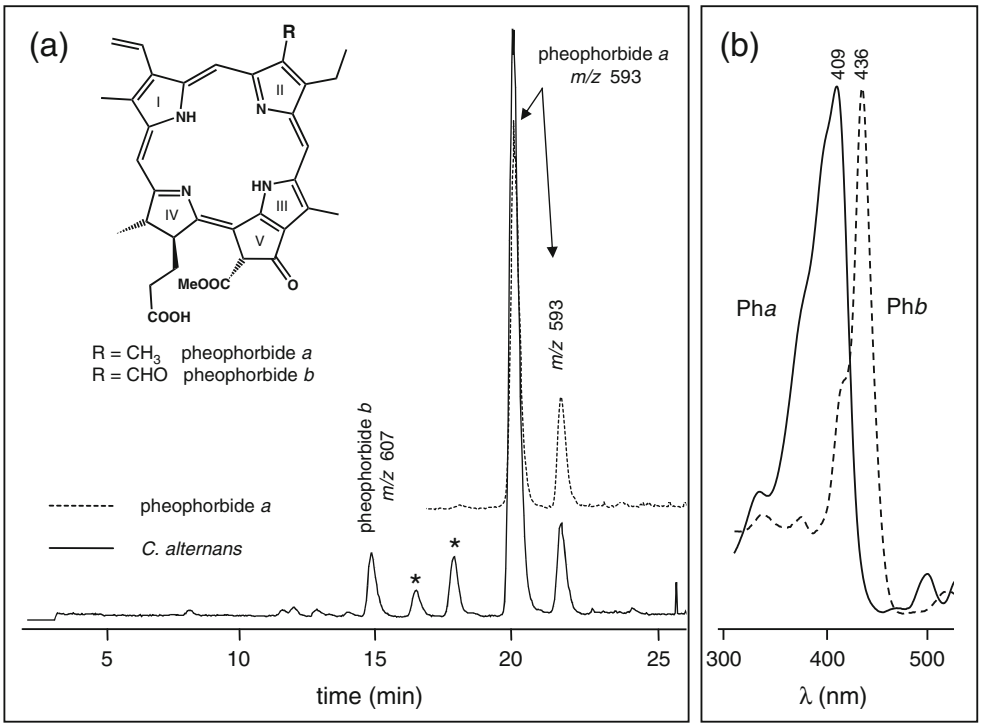

Fig. 2 Chromatographic separation and identification of chlorophyll catabolites from fecal shields of *C. alternans*. **a** LC-MS profile of the methanolic extract of larval shields (*straight line*) and the pheophorbide *a* standard (*dotted line*). The *two peaks* for Ph*a* represent epimeric esters of ring V (Smith et al. 1985), Ph*a* (R = CH_3), Ph*b* (R = CHO), (*asterisks*) unknown compounds. **b** UV spectra of Ph*a* (*straight line*) and Ph*b* (*dotted line*)

Quantification of Pheophorbide a For direct quantification of the components in the methanolic extract, shields (ca. 50 mg) were placed in methanol (2 ml) and sonified for 2 min. Solids were deposited by centrifugation (20 min at 20×*g*) and 10 µl of the supernatant, a clear liquid, were diluted with the tenfold volume of methanol followed by analysis by HPLC-MS without further purification.

To quantify pheophorbide *a* in shields, a calibration curve (peak area vs. concentration) was calculated by using reversed-phase HPLC-MS and the commercial Ph*a* standard at concentrations of 0.01, 0.10, 1.0, and 2.0 µg ml^{-1} MeOH under the same conditions used for the shield samples. The pheophorbide *a* standard gave two peaks, (84:16, see Fig. 2a) corresponding to the stereoisomers in ring V of Ph*a*, whose areas were added together and averaged (y=671,095,720.3552x; R^2=0.9947).

Statistical Analyses We examined larval capture times in the bioassay by using a partial regression failure-time approach, the Cox proportional hazard model (PROC TPHREG; SAS 2004). In contrast to classical methods, such as analysis of variance (ANOVA) that compare either the total number of captures at the end of the experimental time interval or the average capture time among treatment groups, failure-time methods compare the distributions of capture times throughout the entire bioassay period. Times to the occurrence of an event (e.g., capture of a larva by ants) typically do not meet the distributional assumptions required by traditional parametric approaches. In addition, many of the trials ended before a capture event was recorded (i.e., right-censored data), and the ultimate fate of the larva beyond the bioassay interval was unknown. Due to the exclusion of such right-censored events, fail-time approaches are more appropriate than ANOVA or frequency-based methods to detect time-dependant changes in resistance.

Capture functions were compared by using the Wilcoxon's signed ranks test followed by pair-wise multiple comparisons to determine specific differences between treatment groups (Kalbfleisch and Prentice 1980). Significance levels were corrected with the sequential Bonferroni technique (Dunn-Sidák method; Sokal and Rohlf 1995). This method is less conservative than the standard Bonferroni technique but ensures that an appropriate experiment-wise error rate (α=0.05) is maintained. Predation rates were graphed using the life table approach (PROC LIFETEST; SAS 2004).

Results

HPLC-MS of Shield Extract The LC-MS of a methanolic extract from *C. alternans* shields revealed two peaks at 20.18 min (84%) and 21.97 min (16%) both with a molecular ion at *m/z* 593.4 (Fig. 2a). Both peaks had a strong UV absorption around 409 nm accompanied by a smaller absorption at 666 nm (Fig. 2b). These data suggested the presence of a porphyrin-type compound, such as pheophorbide *a* (Ph*a*), the major catabolite of chlorophyll *a* in plants. Injection of authentic Ph*a* generated

the same two peaks at 20.10 min (84%) and 21.89 min (16%) in a similar ratio (Fig. 2a). Thus, on the basis of spectral (MS, UV, ^{1}H and ^{13}C nuclear magnetic resonance; see Smith et al. 1985) and chromatographic coincidence, the two major peaks at *m/z* 593 could be attributed to the chlorophyll catabolite, Ph*a* ($C_{35}H_{36}N_4O_5$).

The two 593 peaks are formed by the separation of the epimeric esters in ring V, which resulted from the facile enolization of the β-keto ester into a conjugated system followed by reprotonation. Due to the presence of a second chiral center in ring III, a mixture of diastereomers is formed that readily separates on the chromatographic column. In addition to the two Ph*a* peaks, we identified a minor signal for pheophorbide *b* (Ph*b*) at 14.79 min. Pheophorbide *b* is characterized by a molecular ion at *m/z* 607 and a long-wave UV maximum at 436 nm, which is due to the extended chromophore with the aldehyde group in ring II (Fig. 2a; R = CHO). The signals at 16.4 min (*m/z* 609) and 17.8 min (*m/z* 625) could not be identified (denoted *; Fig. 2a). These peaks most likely represent other, still unknown chlorophyll catabolites, since their UV spectra also exhibited maxima at 410 and 400 nm, respectively. The natural concentration of Ph*a* in *C. alternans* was determined to be 2.38 μg mg^{-1} of fecal shield. An individual shield had a mean (±SE) dry weight of 64 (±4) mg (*N*=34). The naturally relevant Ph*a* shield concentration was, therefore, estimated to be 152.3±9 μg per intact shield.

Bioassay of Pha-Loaded Shields The regression coefficients derived from the Cox proportional hazard model for shields augmented with 3, 5, and 10 μg concentrations of Ph*a* resisted capture significantly longer than did the solvent-leached controls (Fig. 3; *P*=0.05 after Bonferroni correction for multiple pair-wise comparisons; see "Materials and Methods"). Larvae with shields enhanced with 5 μg Ph*a* withstood capture at a slightly lower but not significantly different rate than did larvae with intact shields (Fig. 3). Capture rates for shields with Ph*a* concentrations of 10 μg did not differ significantly from intact shields (*P*>0.05). Shield concentrations of 1 μg of Ph*a* did not differ from solvent control shields (*P*>0.05).

Most of the ants first coming into contact with a treated larva appeared to hesitate, retreat a short distance with their abdomens raised, and then to re-contact the larvae by antennation or by touching (not biting) with open mandibles. Within the first minute of many bioassay trials (77/166) with intact and Ph*a*-treated larval shields, the first ants to contact a treatment larva either retreated a short distance and began antennal grooming, or they repeatedly ran in circles nearby the larva, only to return briefly without eliciting recruits or seizing the larva. In some trials (17/166), ants making initial contact with a Ph*a*-treated larva withdrew directly and never returned or recruited other ants. All of these behaviors created delays and indicated a reluctance to seize a larva. Such hesitancy contributed to

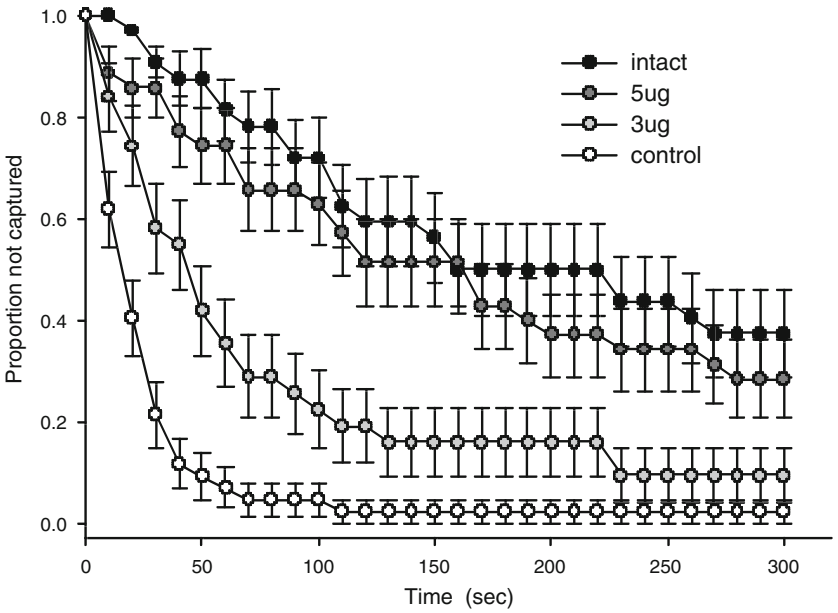

Fig. 3 Survival curves showing capture rates of *C. alternans* larvae with intact, pheophorbide *a*-augmented, or methanol control shields in the *Azteca* ant bioassay. Leached shields were enhanced with either 3 μg/shield (*grey circles*) or 5 μg/shield (*dark grey circles*) concentrations of pheophorbide *a*. Larval controls (*open circles*) had their shields leached (methanol and water), modified with 50 μl of MeOH, and then reattached. Intact shields (*solid circles*) were removed and reattached to larvae without leaching or the addition of any substances. Data from the 1 μg and 10 μg additions of Ph*a* were excluded for the sake of clarity. The former treatment was not different from zero Ph*a*, and the latter treatment did not differ from larvae with intact shields. *Bars* are the standard error of the life table estimate. Samples sizes: 3 μg=36, 5 μg=34, solvent control=41, and intact shields=32

the significantly longer capture times for both the Ph*a* and intact shield treatments. None of these behaviors were observed with the solvent-leached treatments.

Discussion

Our findings demonstrate that the shields of *C. alternans* larvae function mainly as deterrent chemical defenses. Ants avoid larvae only after coming into direct contact with them. On the basis of spectral and structural evidence, we identified actively deterrent compounds that reside in the methanolic extract of *C. alternans* shields as the chlorophyll catabolites, pheophorbide *a*, and pheophorbide *b*. The major metabolite, Ph*a*, with approximately 78% of total chlorophyll catabolites, exhibited significant deterrence against *A. lacrymosa* ants in the field bioassay. Judging from the behavioral responses of *A. lacrymosa*, shields derive their deterrent activity by contact irritancy and then by eliciting disorientation.

Pheophorbide *a* is potent. Deterrence was detected at only 1% or 2% of its natural shield concentration, which is on the order of 100 μg per shield. At 4% of its natural concentration (5 μg), Ph*a* appears to be responsible for over 90% of shield deterrence measured in this type of bioassay against this particular predatory enemy.

The origin of pheophorbide shield derivatives represents a two-step sequestration strategy, consisting of the assimilation and modification of the host plant precursor, chlorophyll, by the insect. Pheophorbide *a* and Ph*b* are most likely produced in the beetle's digestive system from ingested chlorophyll *a* and *b*. Probably no specialized enzymes, besides the ubiquitous lipases and esterases of the gut, are needed to remove the phytol side chain and the central magnesium ion (Kräutler et al. 1997). Pheophorbide *a*, thus, has a unique role in ecology: it is the first time that a chlorophyll degradation product has been assigned an interspecific, non-nutritive defense function, which may have implications for the wider occurrence of chlorophyll degradation products in other defense systems.

Although the vast majority of reports about its activity stem from the mammal literature, Ph*a* appears to have a wide range of activities, including anti-tumor, immunosuppressant, and bio-toxic properties. For example, exposure of human lymphoid leukemia Molt 4B cells to Ph*a* led both to growth inhibition and to the induction of programmed cell death (apoptosis; Hibasami et al. 2000; Chan et al. 2006). Chee et al. (2005) demonstrated that Ph*a* and its derivatives exhibit photocytotoxic activity in human leukemia and oral squamous carcinoma cell lines. As a possible therapeutic, Ph*a* isolated from *Psychotria acuminata* (Rubiaceae) inactivated cell surface receptors and contributed not only to the antitumor effects of photodynamic therapy but also to systematic immuno-suppression (Glinski et al. 1995). As a possible insect toxin, Ph*a* was shown to inhibit cholesterol acyltransferase, an enzyme necessary for the assimilation of the plant sterols that are required by insects to synthesize developmental hormones such as ecdysone (Song et al. 2002). When photosensitized, Ph*a* disrupted mitochondrial electron transport (Kim et al. 2004). Such findings suggest that bioassay experiments conducted in darkness may yield different results. In this connection, it is noteworthy that *C. alternans* larvae feed out of direct sunlight on the abaxial leaf surface. We do not know how the insect protects itself from the effects of Ph*a*. Perhaps the polarity of the molecule, although moderate, contributes to its compartmentalization in the gut, thus insulating it from vulnerable tissues. In favor of this idea is the fact that there is a free carboxyl group that might occur as a salt if the gut pH were alkaline. At least for Lepidoptera, where the foregut is extremely alkaline, with a pH of about 10.5, there are conditions favorable for salt formation (Funke et al. 2008). Future research on events in the gut is required to clarify this question.

Most chemically enhanced shield defenses examined so far rely mainly on host-derived secondary metabolites for their repellence or deterrence (see Müller and Hilker 2004). For instance, the *Cordia*-feeding *Eurypedus nigrosignata* and *Coptocycla leprosa* have potent chemical defenses based on host-derived terpenes (Gómez 1997; Gómez et al. 1999). However, there are a few reports of defenses in larval herbivores attributed to derivatives of co-opted primary compounds, such as chlorophyll or fatty acids. The shields of the *Solanum*-feeding tortoise beetle, *Plagiometriona clavata*, for instance, incorporate both primary host metabolites phytol, hexadecanoic, linolenic and oleic acids, and secondary metabolites like saponins and steroidal alkaloids that mount an effective deterrent defense (Morton and Vencl 1998; Vencl and Morton 1999; Vencl et al. 1999).

By intentionally removing behavior from the defense equation, this study focused on compounds that were separated with LC-MS, and the main peaks detected turned out to be potent in the *Azteca* ant bioassay. Since immobilized larvae with intact shields used in this study were not completely protected from *Azteca* ants—60% were taken within the 5-min bioassay timeframe—there may be more to the *Chelymorpha* defense, namely, (1) other shield chemicals, and (2) evasive behaviors and shield waving. First, from a theoretical standpoint, an herbivore that is threatened by many different enemies should incorporate a variety of different defensive compounds, rather than relying on a single repellent or deterrent, which may have limited efficacy against a broad spectrum of enemies. Even assuming that *Azteca* presently is and has been the major larval enemy, the possibility exists that there are other shield compounds, which may target different

enemies. Interestingly, phytol, a chlorophyll accessory molecule, along with a restricted suite of fatty acids, all of which are demonstrably deterrent (see references above), were detected by GC-MS examination of the hexane extract of *C. alternans*' shields (unpublished data). Future bioassay work will be required to determine if these ancillary compounds add to *C. alternans*' shield defense against *Azteca* or perhaps other enemies as well. Second, a live larva with intact behavior should survive this bioassay significantly better. For instance, a previous study that compared live *Acromis sparsa* and *C. alternans* larvae feeding on the same host (thereby equalizing chemical input to their shields) found that both species were equally resistant to *Azteca* ant predation (Vencl et al. 2005). Compared to *A. sparsa* however, *C. alternans* larvae responded to ant attack with increased defensive behaviors (escape and shield-waving). Moreover, once *C. alternans* larvae were deprived of such behavioral tactics (by freezing), they became significantly more vulnerable to predation compared to their equally immobilized counterparts. The resistance of live *C. alternans*, thus, appears due to higher levels of behavioral defenses. The ability to flee quickly, to tightly grip the substrate, and to reduce their apparency by hiding are important defense tactics for many Cassidinae species as well as for other exophytic larvae, such as those of the Lepidoptera (Stamp and Bowers 1992; Gentry and Dyer 2002; Zalucki et al. 2002). Future avenues of research should assess the importance of chemistry in relation to other defenses to first evaluate their separate contributions and then their possible interactions that may enhance overall survival in a nonlinear fashion.

Over the past 50 years, numerous studies have documented the role sequestered plant secondary metabolites play in herbivorous insect defenses against predators and parasitoids (Whitman et al. 1990; Trigo 2000; Nishida 2002). Given their astonishing diversity in plants, it is surprising that the majority of known defensive phytochemicals utilized by larval herbivores is restricted largely to the derivatives of just three secondary compound classes: phenolics, terpenes, and nitrogen-containing compounds, such as alkaloids and amino acids (Harborne et al. 1999; reviewed by Nishida 2002). This discrepancy may be due partly to an insufficient survey of larval defenses, especially of fore and aft discharges, many of which may rely on the assimilated secondary metabolites, and as demonstrated here, the degradation products of host-derived primary precursors. Erstwhile nutrients like Ph*a*, when activated and positioned strategically, may represent an economical and less dangerous alternative to sequestration of potentially auto-toxic or costly endogenously synthesized defense compounds. The widespread correlation of elaborate morphological annexes, fecal retention behaviors with appropriated host plant chemistry, strongly supports the idea that the recurrence of these traits constitutes an under-recognized, multi-trait integrated phenotype. It is conceivable that such trait assemblages may have been responsible for the astonishing success of the limited number of herbivore lineages whose exophytic larvae feed in the dangerous leaf adaptive precinct.

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Hyperaccumulators and Herbivores—A Bayesian Meta-Analysis of Feeding Choice Trials

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Abstract The leading hypothesis for the evolution of the metal hyperaccumulation trait in plants is as a defense against herbivores. A central piece of evidence expected for this hypothesis is that plants benefit from herbivores being deterred from eating high metal tissues. While many studies have investigated whether or not herbivores are deterred by high metal feeds, there has been no quantitative synthesis of these studies. We performed a Bayesian meta-analysis of 31 feeding choice trials from ten published studies, where invertebrates were offered diets of plant tissue from hyperaccumulating species with high and low metal concentrations. Results of individual trials ranged from distinct preference to distinct aversion for high metal diets. The overall mean effect was for herbivore aversion to high metal diets, whether we used fixed or random effects. However, random effect models were better supported than fixed effect models, indicating there was much real variation between trials. This variation could be attributed partly to each of herbivores, plants, studies, and metals, with herbivores being the greatest source of variation. On average, high metal diets deterred insects but not gastropods, which is supported by other research of metal tolerance and sequestration by gastropods. This suggests that the evolution of hyperaccumulation may have differing selective pressures depending upon the suite of herbivores the plants are naturally exposed to. Future studies should give greater consideration to the selection of herbivores and plants tested.

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Introduction

Metal hyperaccumulation, i.e., the ability to accumulate and tolerate large concentrations of metal[1] in aboveground tissues, has been identified in over 415 species of plants across a range of genera, families, and orders worldwide (Baker et al. 2000). Threshold concentrations for the metal hyperaccumulation trait are orders of magnitude higher than the concentrations found in "normal" plant species, e.g., the thresholds for classification as a zinc, nickel, or selenium hyperaccumulator are 10,000, 1,000, or 100 μg g^{-1} metal in the shoots (dry weight), respectively (Baker et al. 2000; Reeves and Baker 2000). Interest in metal hyperaccumulating plants is high within both the research and management communities because of the potential use of hyperaccumulating species in phytoremediation of metal-contaminated sites and the need to conserve the unique and often endangered metalliferous ecosystems in which hyperaccumulating species are found (Whiting et al. 2004).

Boyd and Martens (1992) evaluated early research on hypotheses for the evolution of hyperaccumulator plants and concluded that the herbivory/pathogen-defense hypothesis was the most plausible. Their review stimulated much research that explored the defensive role of hyperaccumulated metals against terrestrial invertebrate herbivores. Boyd's review of 34 studies of the defense hypothesis

[1] Metal hyperaccumulating plants generally are considered to include those that hyperaccumulate metals, such as zinc or copper, and/or metalloids, such as selenium or boron. Throughout this paper, the term metal is used to include both metals and metalloids.

(Boyd 2007) has clarified the categories of evidence needed for demonstration of a selective benefit of hyperaccumulation. There is considerable, but not total, support for the herbivory-defense hypothesis among the hyperaccumulator research-community (e.g., Boyd et al. 2002; Jhee et al. 1999; Pollard 2000; Pollard and Baker 1997). One form of evidence for the herbivory-defense hypothesis is herbivore deterrence in feeding choice trials (Boyd 2007). A number of researchers have investigated the potential of hyperaccumulated metals to protect plants from herbivory via feeding-choice experiments where invertebrate herbivores were offered a choice of leaf tissue (high or low metal concentration) from hyperaccumulator plants, and the feeding preferences were analyzed. Results have varied widely among these, ranging from aversion (e.g., Boyd et al. 2002; Hanson et al. 2004; Martens and Boyd 2002) to preference for a high metal diet (e.g., Boyd and Martens 1999; Hanson et al. 2003; Noret et al. 2005). Findings from choice trials were tabulated, reporting the number that showed a defensive effect (Boyd 2007). This methodology, called vote counting, is limited in its utility for quantitative synthesis (Hedges and Olkin 1980; Bushman 1994). Specifically, Boyd (2007) did not examine the magnitude of the effect and variation therein. By building on Boyd's review (2007), we report here a Bayesian meta-analysis of the published data from feeding-choice trials. Our objective was to investigate quantitatively the following questions: (1) Is there a deterrent effect of high metal concentrations in the leaf tissue of hyperaccumulator plants against herbivory by terrestrial invertebrates? (2) If so, how large is the effect? (3) How does the effect vary among trials? (4) Can the variation be explained?

Methods and Materials

Meta-analysis is a process of quantitative synthesis of results of multiple studies of the same process. By combining results of multiple studies, greater statistical power to examine hypotheses is attained. Moreover, the generality of a model can be examined and effects of covariates determined. We used a Bayesian statistical framework for our analysis for two main reasons. First, we were interested in estimating effect sizes or parameters and confidence in them, rather than the statistical significance of a test of a null hypothesis, as what occurs with classical or frequentist statistical approaches (McCarthy 2007). Second, in many cases, random effect models are more appropriate than fixed effects models (Raudenbush 1994). This is because we expect variation of parameters for biological and logistic reasons, i.e., a range of effect sizes, due to differences in biological interactions that are beyond sampling variation. The technical aspects of performing frequentist random effects meta-analyses are complex, and procedures are neither straightforward nor accepted universally (Raudenbush 1994). By contrast, random effect models, and models with a mixture of fixed and random effects, can easily be constructed and evaluated with Bayesian inference by using Markov Chain Monte Carlo sampling methods (Clark 2005; Link et al. 2002). For an introduction to Bayesian modeling, we suggest Link et al. (2002) and McCarthy (2007).

Our aim here was to compile quantitatively and analyze a data set of invertebrate binary choice feeding trials. We obtained the data set from peer-reviewed publications that reported feeding choice trials of invertebrate herbivores offered control and high metal concentration diets of metal hyperaccumulating plants. We located the publications through personal bibliographies, searches of the online database, Thomson ISI Web of Science (latest search April 2008), and the tabulation provided by a recent review (Boyd 2007). Ten papers were found that fit our criteria for analysis, namely, that means, sample sizes, and precisions (standard errors) for consumption of plant tissue were accessible in the paper. Multiple trials often were reported in a single publication, either as repeat runs or by using different herbivores or plant population combinations. Thus, our data set comprised 31 individual trials (Table 1).

To account for differences in the way trials were conducted (e.g., age and size of herbivores, trial duration) and how response variables were measured (e.g., area vs. mass consumed, absolute vs. relative consumption), we transformed the response data to reflect the proportional change in consumption between control and high metal diets. Because the minimum consumption of a herbivore is zero and because most individuals ate small amounts with a few individuals eating large amounts (i.e., a right skewed distribution), we modeled the data with log-normal distributions. In addition, the skewed data led to standard errors that increased in magnitude with the mean. Hence, we used means and standard errors as presented in the original papers to calculate the parameters for log-normal distributions by using the following formulae (McCarthy 2007):

$$c = \sigma^2/\mu^2 + 1, \tag{1}$$

$$a = 1\mathrm{n}(\mu) - 0.51\mathrm{n}(c), \tag{2}$$

$$t = 1/1\mathrm{n}(c), \tag{3},$$

where μ is the reported mean, and σ the reported standard error of the mean, c is a scaling parameter for the conversion, a is the mean of the log-normal, and t is the

Table 1 Summary of 31 binary choice feeding trials of invertebrate herbivores offered high and low metal concentration diets from metal hyperaccumulator plants

Source	Plant	Herbivore	E	τ	Metal	Trial no.
(Boyd et al. 2002)	*Senecio coronatus*	*Helix aspersa* (G)	−0.14	4.00	Ni	22
(Boyd et al. 2002)	*Senecio coronatus*	*Helix aspersa* (G)	2.04	1.30	Ni	8
(Boyd et al. 2002)	*Senecio coronatus*	*Helix aspersa* (G)	2.41	2.20	Ni	7
(Hanson et al. 2003)	*Brassica juncea*	*Mesodon ferrissi* (G)	−2.49	1.89	Se	31
(Hanson et al. 2003)	*Brassica juncea*	*Mesodon ferrissi* (G)	−1.15	10.88	Se	27
(Hanson et al. 2003)	*Brassica juncea*	*Mesodon ferrissi* (G)	−0.48	46.15	Se	25
(Hanson et al. 2003)	*Brassica juncea*	*Pieris rapae* (I)	3.01	1.47	Se	4
(Jhee et al. 2005)	*Streptanthus polygaloides*	*Melanoplus femurrubrum* (I)	1.86	6.02	Ni	9
(Jhee et al. 1999)	*Thlaspi caerulescens*	*Pieris napi oleracea* (I)	0.27	34.18	Zn	19
(Jhee et al. 1999)	*Thlaspi caerulescens*	*Pieris napi oleracea* (I)	1.01	43.73	Zn	13
(Martens and Boyd 1994)	*Streptanthus polygaloides*	*Pieris rapae* (I)	0.50	14.65	Ni	18
(Noret et al. 2005)	*Thlaspi caerulescens*	*Helix aspersa* (G)	−0.38	3.08	Zn	24
(Noret et al. 2005)	*Thlaspi caerulescens*	*Helix aspersa* (G)	1.29	3.22	Zn	11
(Noret et al. 2005)	*Thlaspi caerulescens*	*Helix aspersa* (G)	−2.14	4.33	Zn	30
(Noret et al. 2005)	*Thlaspi caerulescens*	*Helix aspersa* (G)	−0.91	8.32	Zn	26
(Noret et al. 2005)	*Thlaspi caerulescens*	*Helix aspersa* (G)	0.91	15.79	Zn	14
(Noret et al. 2005)	*Thlaspi caerulescens*	*Helix aspersa* (G)	−1.33	3.26	Zn	28
(Noret et al. 2005)	*Thlaspi caerulescens*	*Helix aspersa* (G)	−1.76	4.64	Zn	29
(Noret et al. 2005)	*Thlaspi caerulescens*	*Helix aspersa* (G)	−0.02	15.57	Zn	20
(Noret et al. 2005)	*Thlaspi caerulescens*	*Helix aspersa* (G)	0.80	11.24	Zn	15
(Noret et al. 2005)	*Thlaspi caerulescens*	*Helix aspersa* (G)	−0.13	5.42	Zn	21
(Noret et al. 2005)	*Thlaspi caerulescens*	*Helix aspersa* (G)	0.56	6.05	Zn	17
(Pollard and Baker 1997)	*Thlaspi caerulescens*	*Deroceras caruanae* (G)	1.57	15.42	Zn	10
(Pollard and Baker 1997)	*Thlaspi caerulescens*	*Pieris brassicae* (I)	4.39	1.4	Zn	3
(Pollard and Baker 1997)	*Thlaspi caerulescens*	*Schistocerca gregaria* (I)	1.28	9.81	Zn	12
(Behmer et al. 2005)	*Thlaspi caerulescens*	*Schistocerca gregaria* (I)	0.73	20.95	Zn	16
(Freeman et al. 2006)	*Stanleya pinnata*	*Pieris rapae* (I)	6.79	0.22	Se	1
(Freeman et al. 2006)	*Stanleya pinnata*	*Plutella xylostella* Stanleyi (I)	−0.19	5.17	Se	23
(Freeman et al. 2006)	*Stanleya pinnata*	*Plutella xylostella* G88 (I)	5.35	0.31	Se	2
(Freeman et al. 2007)	*Stanleya pinnata*	*Orthoptera* spp. (I)	2.88	4.56	Se	5
(Freeman et al. 2007)	*Stanleya pinnata*	*Orthoptera* spp. (I)	2.50	7.12	Se	6

E is the effect size and is positive where control diets were preferred to high metal diet. τ is the precision of the estimated effect size and is the inverse of the variance. See text for details. Trial refers to the trial number in Fig. 1. *G* after the herbivore indicates gastropod, *I* indicates insect

precision (inverse of the variance) of the log-normal. Where data were only presented as graphs, we first scanned the figures and then used the program Datathief (http://www.datathief.org/) to recover mean and standard error values. The effect size (E) of the metal on consumption was then calculated as:

$$E = a_{\text{control}} - a_{\text{metal}} \quad (4).$$

The precision of the effect size was calculated as (assuming independence, so that the variances are additive):

$$\tau = \frac{1}{\left(\frac{1}{t_{\text{metal}}} + \frac{1}{t_{\text{control}}}\right)} \quad (5).$$

In this way, we obtained the basic dataset where the response variable was the effect size, E, and associated precision, τ, for each binary choice trial (Table 1).Note that these effect sizes are not *differences* but *differentials* that reflect how many times more of one diet is consumed than another. Because the effect is on a natural log scale, values for E of 0.5, 1, 2, 3 correspond to eating 1.6, 2.7, 7.4, and 20 times as much of the control diet over the high metal diet; negative values of E imply preference for the high metal diet. τ is the inverse of the variance and, thus, can be converted to a standard error by taking the square root of the inverse. Large values of τ equal small standard errors; e.g., τ =0.5, 1, 5, and 10 yield standard errors of 1.4, 1, 0.45, and 0.32, respectively.

The differentials obtained above were modeled with simple linear models by using Bayesian statistics. Models were fitted by using the Bayesian statistical modeling software WINBUGS 1.4.1 (Lunn et al. 2000), available as freeware from http://www.mrcbsu.cam.ac.uk/bugs/. We began by modeling the differential as a fixed coefficient, such that observed effect sizes were distributed around the grand mean with only random error variance. Next, we modeled the differential as a random coefficient, such that observed effect sizes were random draws from a population of effect sizes for which we inferred the mean and variance. This allowed all trials to have their own effect size, which is drawn from some unknown distribution that we seek to model. Third, we sought to examine the effect of introducing information about covariates as random effects into the models with the differential being a fixed effect or a random effect. Four covariates were included: (1) the publication; (2) the plant species (one of *Brassica juncea*, *Senecio coronatus*, *Stanleya pinnata*, *Streptanthus polygaloides*, *Thlaspi caerulescens*); (3) the herbivore species (see Table 1); and (4) the metal studied (nickel, selenium, or zinc). Finally, we fitted a model with an herbivore type as fixed effect to examine the difference between gastropods and insects.

Although Bayesian inference has the potential to incorporate prior information as expert belief or ancillary data, we chose not to use this, and rather used minimally informative prior distributions for the parameters of interest. All fixed effect coefficients were modeled as being drawn from normal distributions with mean 0.0 and SD 1,000. Random effect coefficients were modeled as normally distributed with mean 0.0 and SDs drawn from a uniform distribution over the interval (0, 100). Three Markov chain Monte Carlo (MCMC) chains with different initial values (−1, 0, 1) were run simultaneously for each model. In all cases, burn-in phases were >1,000 iterations. Standard Bayesian MCMC diagnostics were used to assess convergence, including plots of each of the chain traces, chain autocorrelations, and the Gelman–Rubin statistic (Spiegelhalter et al. 1995; Zuur et al. 2002). Bayesian 95% credible intervals were calculated from the posterior probability density of monitored parameters, in each case by using >50,000 samples. When minimally informative priors are used, as used here, credible intervals are practically equivalent to frequentist confidence intervals calculated with maximum likelihood estimation. The estimates for the effect sizes (E) (and credible intervals) were then converted back to an arithmetic scale by exponentiating, i.e., differential consumption=e^E.

For model selection, the series of models was compared by using the deviance information criterion (DIC) (Spiegelhalter et al. 2002), calculated with WINBUGS. In a given dataset, the model with the lowest DIC is the model that would be most likely to produce a dataset of the same structure as that observed. That is, it is the model that best balances model fit, as measured by the deviance, and model complexity, as measured by the estimated number of parameters in the model. The percentage of deviance explained by a fitted model (similar to R^2) was calculated as the total deviance minus the deviance for the fitted model, divided by the total deviance, using the deviances at the posterior mean of the parameters $\left(\hat{D}\right)$.

Results

The effect sizes (E) modeled for individual trials ranged widely from strong aversion to strong preference (Fig. 1). For the null model, which posits that all measured effect

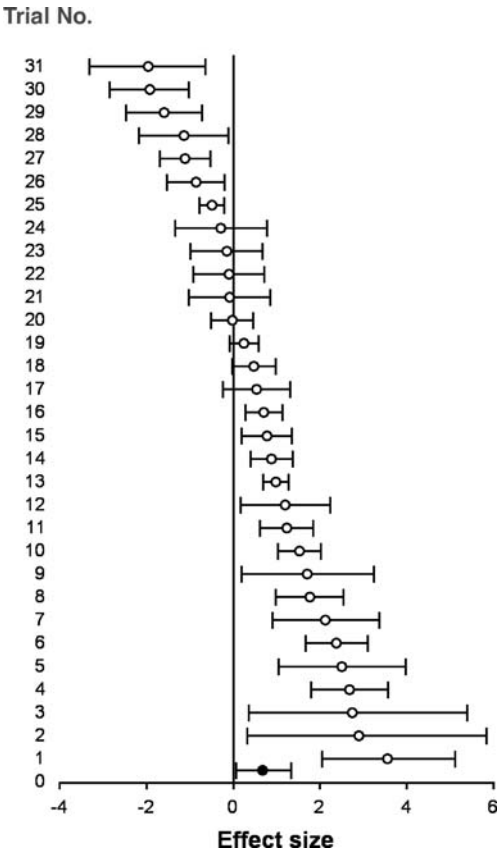

Fig. 1 Effect sizes (with 95% Bayesian credible intervals) of 31 binary choice trials of invertebrate herbivores offered control and high metal concentration plant tissue from a random effects model. Effect size is a natural log scale; positive values indicate aversion to the high metal, and negative ones indicate preference. *Zero* indicates no preference, equal amounts of control and high metal diets are chosen. Individual effect sizes of trials (*open circles*) range widely from strong preference to strong aversion to the metal (see Table 1 for identification of the trial the numbers refer to). The credible interval around the mean effect size (*filled circle, at bottom of graph*) reflects uncertainty about the location of the mean. The distribution of 95% of population effect sizes expected is about twice as wide

sizes should be equal and only vary through sampling error, the mean effect size (and 95% credible interval) was 0.43 (0.32, 0.54). This implies that, on average, invertebrates avoided the high metal plant leaves and ate a mean of 1.54 (1.38–1.72) times as much control diet as high metal diet. However, compared by DIC, the fixed effect model was the poorest of those examined (Table 2). The random effects model had a mean effect size of 0.71 (0.10, 1.36; Fig. 1) corresponding to herbivores eating 2.03 (1.10, 3.89) times as much control diet as high metal diet. The SD of the estimated mean effect was 0.32, but that among trials was fivefold greater, 1.62 (1.19, 2.29), which underscores the relevance of trial-to-trial variation. In other words, the mean effect is larger, but less certain when we account for real variation among trials.

Introduction of covariates improved fixed effect models beyond the null model (see model 11 in Table 2). This is because, for a given level of a covariate, e.g., for the herbivore *Pieris rapae*, the observed effect sizes from different trials were far from equal. In comparison, once the random effect of the trials was included, the addition or removal of other covariates had relatively little influence on model selection (Table 2). Models that included herbivore or plant were better supported than those that included metal or study. There was little evidence for an effect of metal type. The effects for the individual metals all had SDs at least three times as large as the means.

The best-supported model had random effects of herbivore, plant, and trial (Model 1 in Table 2). According to this model, the mean effect size was 1.41 (−0.40, 3.53), corresponding to 4.1 (0.67, 34.1) times as much control food as high metal food being consumed (Fig. 2). Hence, the uncertainty increased over the model of trial-to-trial variation only, and we cannot be certain that, on average, high metals deter herbivores. Examining the SD of the various random effects revealed important variation among herbivores [SD_h=1.63 (0.52, 3.52)], and among plants [SD_p=1.16, (0.10, 4.63)], and variation between trials was reduced [SD_t=1.06 (0.70, 1.69)] relative to the model with a random trial effect only. The uncertainty about variation among plants was in part because so few hyperaccumulators have been studied. On average, *Stanleya pinnata* and

Table 2 Comparison of models of the effect of high metal concentrations in plant tissue on invertebrate consumption

	Model	DIC	$\widehat{D}$	Explained deviance (%)
1	Trial, herbivore, plant	66.0	11.0	96.9
2	Trial, herbivore, study	66.6	11.7	96.7
3	Trial, herbivore, metal	66.8	12.0	96.6
4	Trial, herbivore	67.7	13.2	96.3
5	Trial, plant	68.0	11.8	96.7
6	Trial, study, plant	68.2	13.4	96.2
7	Trial, metal	68.6	12.3	96.5
8	Trial, study	68.8	13.4	96.2
9	Trial	69.0	13.0	96.3
10	Herbivore, plant, study	143.5	115.8	67.4
11	Null model, fixed mean effect only	356.9	354.9	0

DIC is deviance information criterion, $\widehat{D}$ is the deviance at the posterior mean of the parameters, Explained deviance reports how much residual deviance after the fitting of a null model (no. 11) is explained by a particular target model. Models with low DIC values are more parsimonious than those with high ones. Models with DIC values that differ by less than about 2 are of equivalent predictive value. All models that included a random effect of trial were more parsimonious than those that did not. Only one such model that lacked an effect of trial is included here for comparison, model 10

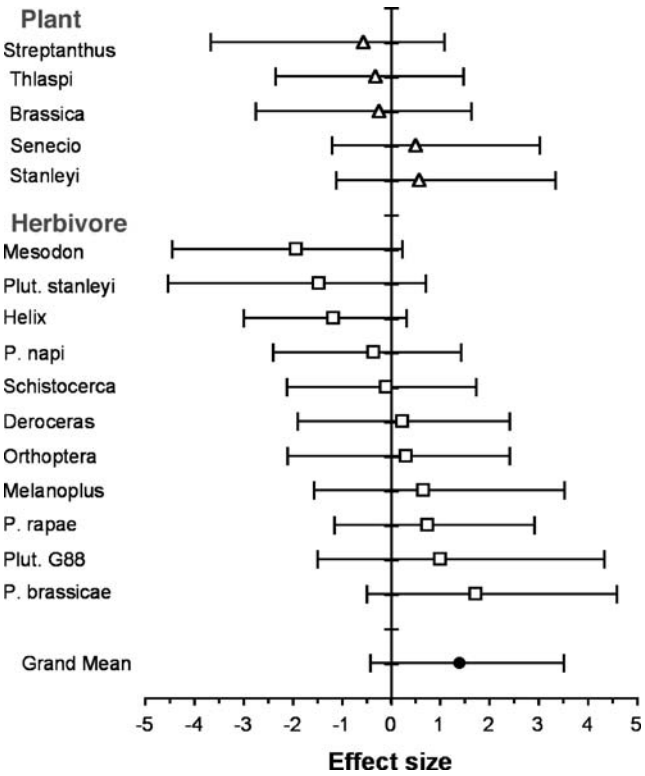

Fig. 2 Plot of the mean (and 95% Bayesian credible intervals) for the effect of plants (*triangles*) and herbivores (*squares*) on differential consumption of control and high metal concentration plant tissue in feeding choice trials. The Grand Mean (*filled circle*) effect is at the bottom of the graph. Zero indicates no preference, equal amounts of control and high metal diets are chosen. The effects of the plants and herbivores are centered on zero and would be added to the Grand Mean. Effect size is a natural log scale; positive values indicate aversion to the high metal, and negative ones indicate preference. Credible intervals here reflect the uncertainty around mean effect sizes. The distribution of 95% population effect sizes expected is about twice as wide

Senecio coronatus tended to evoke greater herbivore deterrence (Fig. 2).

Of the herbivores, the snails, *Mesodon ferrissi* and *Helix aspersa*, were less likely to be deterred by high metal concentrations, thus suggesting a difference in tolerance between gastropods and insects (Fig. 2). Further, the demonstrably Se-tolerant moth, *Plutella xylostella* Stanleyi (Freeman et al. 2006), also was less likely to be deterred by high metal (Se) than other herbivores.

Probing deeper into the herbivore effect, we fitted a model with a fixed effect for herbivore (insect vs. gastropod) as well as random effects for plant and trial (Table 3). This revealed that gastropods were unlikely to be deterred from eating high metal plants; the credible interval around the mean effect for the gastropods included zero, and the mean effect was close to zero (Table 3). By contrast, high metals were likely to deter insects. The contrast between the effects of the herbivores (insects minus gastropods) was 1.84 (0.67, 3.08), which means that the deterrent effect of high metals was on average 6.3 times (1.95, 21.7) greater for insects than for gastropods. Note that the Se-tolerant *Plutella* was included in the insects, yet this did not influence the average deterrence of insects greatly.

Discussion

The meta-analyses revealed three major findings. (1) On average, high metal concentrations in hyperaccumulators deterred herbivory by terrestrial invertebrates. (2) The variation was so great, however, that individual trials revealed results that ranged from distinct metal aversion to distinct metal preference. Some of this variation could be explained by the identity of herbivores, plants, metals, and studies. (3) Herbivores were the greatest source of variation in deterrence, with the suggestion that hyperaccumulation deterred insects but not gastropods.

This work supports an interpretation that metal hyperaccumulation by plants leads to herbivore deterrence, which is a key piece of evidence expected for antiherbivory defense being a reason for the evolution of hyperaccumulation (Boyd 2007). The variability between the effect sizes of individual trials was too great to be considered the result of simple "sampling error". Rather, there is a wide distribution of true effect sizes. It is expected that future studies will report results that range from distinct metal aversion to distinct metal preference, and that some of the range is because there are situations where the true preference is for a high metal diet, rather than just being a result of stochastic sampling variation.

The study identity explained some of the variation in feeding choice. There are a number of possible interpretations of this result. A skeptical interpretation is that experimenters have strong influence on results. A more positive and probable interpretation is that although the herbivore, plant, and metal all influence the effect of high metal on feeding choice, these factors are more similar within studies than between studies. For instance, while three studies compared different herbivores, none compared different plant species. Indeed, one study (Noret et al. 2005) contributed 11 trials, all with the same herbivore and plant species combination. That publication points to a possible "file drawer effect," whereby single or underpowered trials that fail to detect herbivore deterrence are either not submitted for publication or are rejected because they do not match with (unspoken) expectations (Moller and Jennions 2001). The study of Noret et al. (2005) contributed a disproportionate share of trials that indicated preference for high metal diets; perhaps so many trials were needed to convince authors, editors, and reviewers that the findings were not "mistakes." Ecotypic variation within plants and herbivores studied in multiple studies also may contribute to the variation.

Because the distribution of herbivores and plants across studies was limited, uneven, and confounded, the power of

Table 3 Parameter estimates for a model with a fixed effect of insects versus gastropods and random effects for plants and trials

	Mean	SD	2.5% CI	Median	97.5% CI
Insects	1.89	0.72	0.51	1.86	3.42
Insects-Gastropods	1.85	0.61	0.67	1.84	3.08
Gastropods	0.03	0.75	−1.34	0.01	1.53
Brassica	−0.19	0.67	−1.68	−0.11	1.05
Thlaspi	−0.30	0.82	−2.26	−0.14	1.12
Streptanthus	−0.41	0.79	−2.28	−0.25	0.86
Stanleyi	0.53	0.86	−0.79	0.35	2.58
Senecio	0.29	0.77	−1.12	0.17	2.01
SD_p	0.97	1.02	0.04	0.72	3.43
SD_t	1.34	0.24	0.94	1.32	1.89

The fixed effect for the herbivore type was modeled such that gastropods were the reference class. This model had a DIC value of 66.6, and $\hat{D} = 11.9$. Insects–gastropods is the contrast between the two classes of herbivore

the analysis to estimate effects of herbivore or plant or metal was limited. Estimates of mean effect sizes for plants and herbivores varied over threefold and 30-fold, respectively, when back-transformed (Fig. 2), but the credible intervals for predictions were broad. The most striking result was that high metal diets did not deter snail herbivory. Our random effect analysis recovered the effect of the evolution of Se-tolerance in a taxon of *Plutella xylostella* (Freeman et al. 2006; compare the two *Plutella* effects in Fig. 2). Yet, high metals deterred the two snail taxa even less than the Se-tolerant *P. xylostella stanleyi*. Indeed, in a study that we could not include in our analysis because we could not extract the relevant statistics, *Helix aspersa* did not distinguish between low and high metal diets of *Arabidopsis halleri* (Huitson and Macnair 2003), thus supporting our results for snails. We note that snails, particularly the species most frequent in our dataset, *Helix aspersa*, have been shown to be tolerant of, and to accumulate, high levels of metals (Coughtrey and Martin 1976, 1977). Snails also regulate free metal concentrations through binding to metallothioneins and calcium phosphate granules (Berger and Dallinger 1989; Howard et al. 1981; Marigomez et al. 2002). If the two snail species, *Mesodon ferrissi* and *Helix aspersa*, and the Se-tolerant *Plutella xylostella* Stanleyi were omitted from the analysis (e.g., in Fig. 2), the effect would be to increase the grand mean deterrent effect and reduce its uncertainty due to these herbivores being the least deterred by metal-rich diets.

Our fixed effect contrast of the gastropods and insects showed that while insects were deterred from eating high metal plants, gastropods were not. This is an important result for better understanding the evolution of hyperaccumulation as a defense. Caution is required because, strictly, the fixed effect analysis cannot be used as a basis for inference about other gastropods and insects, unlike the random effects analysis. The fixed effect also ignores herbivore species variation within those classes of insects and gastropods. This clearly is incorrect; compare the two snails *Helix aspersa* and *Mesodon ferrissi* with the slug *Deroceras caruanae* (Fig. 2). However, visual interpretation of the parameter estimates from the random effect model in Fig. 2 supports the finding that, on average, hyperaccumulation deters insects but not gastropods. Whether this is a general result requires targeted research. Our analyses suggest the evolution of hyperaccumulation may have differing selective pressures that depend upon the suite of herbivores the plants are naturally exposed to. Where gastropods are a large component of potential herbivores, selection for hyperaccumulation as a defense may be diminished.

Explanation for the wide variability in the results may lie in the choice of widespread, generalist herbivores for most herbivory-defense trials on hyperaccumulating plants, including those analyzed here. Herbivores endemic to, or at least occurring in, areas inhabited by hyperaccumulator species may be better able to identify metals in plant tissues and avoid them. Further, with sufficiently long exposure to hyperaccumulators, one might expect the evolution of deterrence and/or detoxification mechanisms in some of these endemic herbivores (e.g., Freeman et al. 2006; Wall and Boyd 2006). We suggest that future researchers pay more attention to the choice of plant–herbivore pairings. Studies that cover multiple plant–herbivore combinations reduce the confounding of studies, plants, and herbivores. Further research also could use the results of this work as priors to be updated with new data using the Bayesian statistical framework (McCarthy and Masters 2007).

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Physiological, Nutritional, and Biochemical Bases of Corn Resistance to Foliage-Feeding Fall Armyworm

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Abstract Three corn (*Zea mays*) germplasm lines [i.e., Ab24E (susceptible control), Mp708 (resistant control), and a locally selected partial inbred line FAW7050 (resistant)] were examined for *Spodoptera frugiperda* (J.E. Smith; Lepidoptera: Noctuidae) resistance. Nutritional [i.e., total protein content, amino acids, glucose, total nonstructural carbohydrates (TNC), protein to TNC (P/C) ratios] and biochemical (i.e., peroxidase and lipoxygenase 3) properties in the seedlings of these corn lines were examined to categorize resistance mechanisms to *S. frugiperda.* Physiological changes in photosynthetic rates also were examined in an attempt to explain nutritional and biochemical dynamics among corn germplasm lines and between insect-infested and noninfested corn plants within a germplasm line. Results indicated that *S. frugiperda* larvae survived better and developed faster in susceptible Ab24E than in resistant FAW7050 or Mp708. The three germplasm lines differed in resistance mechanisms to *S. frugiperda*, and the observed patterns of resistance were probably collective results of the P/C ratio and defensive proteins. That is, the susceptibility of Ab24E to *S. frugiperda* was due to a high P/C ratio and a low level of induced defensive compounds in response to insect herbivory, while the resistance of FAW7050 resulted from elevated defensive proteins following insect herbivory, low P/C ratio, and elevated defensive proteins in Mp708 contributed to its resistance to *S. frugiperda*. The elevated protein amounts in resistant Mp708 and FAW7050 following *S. frugiperda* injury were likely due to greater conversion of photosynthates to defensive proteins following the greater photosynthetic rates in these entries. Greater photosynthetic capacity in Mp708 and FAW7050 also led to higher amino acid and glucose contents in these two lines. Neither amino acid nor lipoxygenase 3 played a critical role in corn resistance to *S. frugiperda*. However, high inducibility of peroxidase may be an indicator of *S. frugiperda* susceptibility as observed elsewhere.

Keywords Total protein content · Amino acid · Glucose · Total nonstructural carbohydrate (TNC) · Peroxidase · Lipoxygenase 3 · *Spodoptera frugiperda* · A/Ci curve · Photosynthesis · Lepidoptera · Noctuidae · Herbivory · Resistance · Mechanisms

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Introduction

Plant nutritional levels and allelochemicals often affect plant suitability and resistance to insect herbivores. Insects that feed on diets or host plants that are high in nitrogen generally have greater growth rates, higher efficiency of conversion of ingested food, and shorter developmental times than when fed on plants with low nitrogen content (Mattson 1980; Woods 1999; Chen et al. 2008). Many herbivorous insects can distinguish qualitatively among host plants or diets and feed and oviposit preferentially on high quality plants (White 1984; Fox et al. 1990; Prudic et al. 2005; Bede et al. 2007; Chen et al. 2008). For instance, larvae and females of beet armyworm, *Spodoptera exigua*, preferred

cotton plants, *Gossypium hirsutum*, with higher nitrogen fertilization for feeding and oviposition, respectively (Chen et al. 2008).

Proteins and amino acids in the diet are the major sources of nitrogen for arthropod growth. Many arthropods have evolved effective strategies to combat the inadequacy of nutrients in their environment (White 1993; Bede et al. 2007). Nutrient balance, in particular, the protein to digestible carbohydrate (P/C) ratio also has been shown to be important for the development of many insects under artificial diet conditions (Simpson and Raubenheimer 1993; Lee et al. 2002; Bede et al. 2007).

Many plants undergo significant biochemical changes following attacks by pathogen and insect herbivores (Hildebrand et al. 1989; Felton et al. 1994; Stout et al. 1994; Bi et al. 1997; Chittoor et al. 1999; Ni et al. 2001; Heng-Moss et al. 2004). For example, increased peroxidase activity in response to pathogen infections was found in rice, *Oryza sativa* L., and cotton, *G. hirsutum* L. (Chittoor et al. 1999). Besides peroxidase, changes in activity triggered by pathogen infection and insect herbivory have been demonstrated in other oxidases and hydrolases such as catalases, polyphenol oxidases, lipoxygenases (linoleate/oxygen 13-oxidoreductase), and esterases (Felton et al. 1994; Duffey and Stout 1996; Ni and Quisenberry 2003). The increases in activity of many of these enzymes are involved in plant resistance by decreasing the nutritive value of host plants (Shukle and Murdock 1983; Felton et al. 1994; Duffey and Stout 1996; Ni et al. 2001; Ni and Quisenberry 2003). For example, corn earworm, *Helicoverpa* zea (Boddie; Lepidoptera: Noctuidae), larval feeding on soybean foliage for 72 h significantly increased lipoxygenase activity compared to undamaged foliage (Felton et al. 1994). The growth of *H. zea* larvae fed on previously damaged leaf tissues was significantly reduced (over 27%) compared to larvae fed on undamaged leaves.

Photosynthesis is a complex, integrated, and dynamic metabolic process that optimizes the use of carbon and nitrogen. It is regulated by the sources (e.g., light and CO_2) and sink (e.g., photo-assimilate, such as sugars; Paul 1981; Paul and Foyer 2001). An understanding of plant photosynthetic capacity may provide insight into dynamic changes of proteins, amino acids, and carbohydrates such as glucose and starch in growing plants.

In this study, we aimed to categorize possible mechanisms of resistance to *Spodoptera frugiperda* from nutritional and biochemical perspectives in three germplasm lines of corn. We examined nutritional components [i.e., total protein content, amino acids, glucose, total nonstructural carbohydrates (TNC), amino acids to TNC ratio] and biochemical (i.e., peroxidases and lipoxygenases) properties of these corn germplasm lines with various degrees of insect resistance in response to *S. frugiperda* injury. Changes in photosynthetic rates and A/Ci and light response curves also were investigated in an attempt to elucidate nutritional and biochemical dynamics among germplasm lines and between insect-damaged and undamaged plants.

Methods and Materials

Corn (*Zea mays*) Plants

Corn plants in the greenhouse were grown in 2-l flower pots filled with sphagnum peat moss (Premier Horticulture Inc., Quakertown, PA, USA) and landscape top soil (Hood Timber Co., Adel, GA, USA) at a ratio of 3:1 as a growing medium. The greenhouse was set at 28±2°C with light/dark (L/D) of 14:10 h. Yardiac vegetable Osmocote slow release fertilizer (N–P–K=14:14:14; Greenville, SC, USA) was mixed with the growing medium according to instructions provided by the manufacturer, and no fertilizer was applied thereafter. Corn plants in the field were grown following agronomic practices for Georgia corn production as described in Ni et al. (2008) on Belflower Research Farm at Tifton, GA, USA. In all greenhouse and field studies, three corn germplasm lines (i.e., Ab24E, Mp708, and FAW7050) were used. Ab24E (Callahan et al. 1992) and Mp708 (Williams et al. 1990) represented the susceptible and resistant controls, respectively. FAW7050 was a partial inbred line derived from FAWCC(C5) (Widstrom et al. 1993) that had been self-pollinated for six generations between 2004 and 2006. Thus, the three entries of the two inbreds (i.e., Ab24E, Mp708) and the partial inbred (i.e., FAW7050) will be referred to as germplasm lines throughout the text.

Fall Armyworm (*S. frugiperda*) Infestation and Development in the Greenhouse

Fall armyworm neonates were obtained from a colony maintained at US Department of Agriculture Agricultural Research Service Crop Protection and Management Research Unit at Tifton, GA, USA. Five neonates were gently placed at the whorl of corn plants by using a camel hair brush when plants were at the six-leaf stage. Control plants without *S. frugiperda* neonates were placed away from the infested plants. Seven days after infestation, surviving *S. frugiperda* larvae were counted and weighed. Larval survival rates were calculated as the number of recovered larvae divided by 5. Larvae were thereafter kept individually on modified Pinto bean diet (Burton 1969) until pupation in an insect rearing room at 28±1°C with L/D= 16:8 h. Pupation of larvae was recorded, and pupal biomass was measured. Ab24E and FAW7050 were replicated six

times, while Mp708 was replicated three times because of limited availability of plants.

S. frugiperda Damage in the Field

To investigate *S. frugiperda* damage under natural conditions, artificial infestation of field-grown corn was conducted. The experimental design was a split plot. The plot was first randomly divided into four main plots, and each main plot has six consecutive rows. Each main plot was further split into three subplots with double rows and randomly assigned to three corn germplasm lines. In each subplot, one of the double rows was infested with 15 *S. frugiperda* neonates when the plants were at the six-leaf stage using the previously described protocol (Davis et al. 1996). The other row was used as uninfested control plants. *S. frugiperda* damage was rated at 7 and 14 days, respectively, after infestation by using a scale of 1–9 according to Davis et al. (1992) and Smith et al. (1994), where 1=no damage or few pinholes, 2=few short holes on several leaves, 3=short holes on several leaves, 4=several leaves with short holes and a few long lesions, 5=several holes with long lesions, 6=several leaves with lesions <2.5 cm, 7=long lesions common on one half of the leaves, 8=long lesions common on one half to two thirds of leaves, and 9=most leaves with long lesions.

Sample Preparation for Protein, Amino Acid, and Carbohydrate Assays

All samples collected for protein, amino acid, and carbohydrate assays were from greenhouse-grown plants. The youngest corn leaves (V7) with leaf collars were collected immediately after removal of *S. frugiperda* larvae and flash frozen in liquid nitrogen. Leaves were transported on ice to the lab and stored at −20°C until the bioassays. The experimental design was a 3 (corn germplasm)×2 (insect-infested and uninfested) factorial design. Six samples from each treatment of Ab24E and FAW7050 were collected, while three samples for each treatment of Mp708 were collected.

Total soluble protein and amino acid extraction followed Bi et al. (2003). Fresh corn leaf tissue was ground in liquid N, weighed, and mixed with 0.1 M ice-cold potassium phosphate buffer (pH 7.0) containing 1% polyvinylpolypyrrolidone in a vortex. The mixture was centrifuged at 10,000×*g* at 4°C for 10 min. The supernatant was used for total protein content determination.

For amino acid and soluble nonstructural carbohydrate (glucose, fructose, and sucrose) extraction, freshly ground leaf tissue was weighed and extracted in 1 ml of 80% ethanol in an 80°C water bath for 8 min. Ethanol extracts were centrifuged at 10,000×*g* at 2°C for 10 min. The supernatant was used for total free amino acid and soluble nonstructural carbohydrate determinations.

Nonsoluble nonstructural carbohydrate (starch) extraction followed Marquis et al. (1997). The pellet after ethanol extraction was dried, weighed, and transferred to test tubes and incubated with 2.5 ml 0.2 M sodium acetate buffer (pH 4.5) in a boiling water bath for 1 h. After cooling to room temperature, 2 ml of acetate buffer and 1 ml of amyloglucosidase (0.5%, *w*/*v*) were added to the mixture, and it was incubated at 55°C overnight. The solution was filtered through Whatman filter papers and diluted as needed. The filtered solution was assayed for glucose content. The starch content was estimated as glucose equivalents.

Protein, Amino Acid, and Carbohydrate Determination

Total protein content was determined with the Bradford protein assay, and amounts were calculated relative to a standard curve established by using bovine serum albumin as a standard. Amino acid content was colorimetrically determined with the cadmium (Cd)–ninhydrin procedure (Doi et al. 1981; Fisher et al. 2001), which is a precise and reliable method for determination of α-amino acids. Plant foliar tissues may contain high NO_3^- (Chen et al. 2008) and NH_4^+. Preliminary experiments indicated that the intervention of these ions with the Cd–ninhydrin method was low, which is consistent with other studies (Doi et al. 1981; Fisher et al. 2001). The working reagent was prepared according to Doi et al. (1981). Briefly, 0.8 g ninhydrin was dissolved in 10 ml acetic acid and 80 ml absolute ethanol (solution 1). One gram of Cd was dissolved in 1 ml deionized water (solution 2). Solution 1 and solution 2 were mixed, forming a stock solution. The working reagent was made by dilution of the stock solution in deionized water with a 1:1.5 ratio. The determination procedure followed Doi et al. (1981) with modification. Fifty microliters of sample supernatant were mixed with 1 ml working reagent in microcentrifuge tubes and heated at 84°C for 10 min. After cooling, the mixture was transferred to a 1.5-ml disposable cuvette with a 1-cm light path (BrandTech Scientific, Inc., Essex, CT, USA). The absorbance was read at 507 nm in the Shimadzu UV Mini-1240 spectrophotometer (Shimadzu Italia, UK) at room temperature against the blank reagent after cooling. The amino acid content was calculated from a standard curve generated by using glycine.

Glucose content was determined following Jones (1979) and the technical bulletin for glucose assay kit (Sigma-Aldrich, St. Louis, MO, USA). The working reagent consisted of 50 mM Tris-HCl buffer (pH 8.0), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 1.5 mM nicotinamide adenine dinucleotide, 1.0 mM adenosine triphosphate, 2 U ml^{-1} of

hexokinase, and 1 U ml^{-1} of glucose-6-phosphate dehydrogenase. Ten microliters of sample (glucose concentration 0.05–5 mg ml^{-1}) were pipetted into 1-cm path cuvette containing 1.0 ml of working reagent and incubated for 15 min at 31°C. The absorbance at 340 nm was determined in the Shimadzu UV Mini-1240 spectrophotometer at room temperature against deionized water. Absorbance of a sample blank (1.0 ml of water+10 µl sample) and a reagent blank (1.0 ml of working reagent+ 10 µl of water) was subtracted from the glucose absorbance. Fructose and sucrose were determined as by Bi et al. (2001) with some modifications. After original glucose determinations, 2 units of phosphoglucose isomerase were added to the cuvette and reincubated for 15 min at 31°C. Fructose content was calculated as the increase in absorbance at 340 nm from the glucose reading. Then 10 µl of 1% (*w*/*v*) invertase were added to a cuvette and reincubated for 15 min at 37°C. The sucrose content was calculated as the increase in absorbance at 340 nm from the fructose reading. Glucose, fructose, and sucrose contents were calculated from a standard curve generated by using D-glucose as a standard. Total nonstructural carbohydrate was estimated as the sum of glucose and starch because the levels of fructose and sucrose were very low. Protein to TNC ratios (w/w) was calculated with mean molecular weight of TNC (equivalent of glucose) as 180.

Peroxidase and Lipoxygenase 3 Activities

Plant samples prepared for total protein content determination were used to assess peroxidase and lipoxygenase 3 (LOX3) activities. The reaction mixture for peroxidase activity was comprised of 300 µl of 20 mM guaiacol, 250 µl of 0.1 M potassium phosphate buffer (0.1 M, pH 6.0), 240 µl deionized water, and 10 µl plant enzyme extract. The reaction was initiated by adding 200 µl of 0.3% hydrogen peroxide. Peroxidase activity was monitored at 470 nm for 2 min in a Shimadzu UV Mini-1240 spectrophotometer at room temperature (approximately 25°C) after initiation of the reaction against a blank control containing all components of the reaction mixture except the enzyme extract. The activity (micromole per minute per milligram protein) was calculated by using the molar extinction coefficient of 26.6 mM^{-1} cm^{-1} for guaiacol at 470 nm. Peroxidase activity of each plant sample was determined from two subsamples, and the mean of the two subsamples was used for statistical analysis to insure the accuracy of the enzyme activity assays.

Protocol for LOX3 was based on the method developed by Axelrod et al. (1981). The reaction mixture consisted of 2.8 ml of a 0.2-M sodium phosphate (pH 6.5) buffer and 200 µl of a 10-mM sodium linoleate buffer. Reaction was initiated by the addition of plant extracts (varying amounts from 1 to 10 µl depending on ΔA/min). Absorbance was monitored at 234 nm for 2 min in the Shimadzu UV Mini-1240 spectrophotometer at room temperature and converted to micromole product per minute per milligram protein by using a molar extinction coefficient of ketodiene (22 mM^{-1} cm^{-1}).

Photosynthesis Evaluation

Physiological responses of corn seedlings (greenhouse grown) were determined 1 week after *S. frugiperda* infestation. All gas-exchange measurements were made from the youngest fully expanded leaf (V7). Photosynthetic rates were measured with a portable photosynthesis system (model LI-6400, Li-Cor, Lincoln, NE, USA). Leaves were illuminated with a light intensity of 1,500 µmol photons m^{-2} s^{-1} generated by the LI-6400. Three types of photosynthetic-related parameters were determined: a survey photosynthetic measurement (to determine photosynthetic rate of experimental plants measured at 1,500 µmol photons m^{-2} s^{-1} and 400 ppm CO_2), a light response curve (using 0, 20, 50, 100, 200, 500, 1,000, 1,500, and 2,000 µmol photons m^{-2} s^{-1} and at 400 ppm CO_2 concentration), and an A/Ci curve (determines light-independent reactions of photosynthesis; rates measured at 1,500 mmol photons m^{-2} s^{-1} light intensity and at CO_2 concentrations ranging from 400, 300, 200, 100, 0, 400, 400, 600, and 800 ppm). Both light and A/Ci curves were measured by an automated program of the LI-6400 capable of generating the desired light intensities or CO_2 concentrations.

Because leaf chlorophylls are the most important photosynthetic pigments of higher plants and are positively correlated to photosynthetic potential (Curran et al. 1990; Filella et al. 1995; Markwell et al. 1995; Richardson et al. 2002), leaf chlorophyll content was measured by using a Minolta SPAD-502 chlorophyll meter (Konica Minolta Sensing, Inc., Japan). The chlorophyll content under field conditions was measured by using SPAD-502 after damage rating. Four plants (replicates) in each row were used for leaf chlorophyll determination. Chlorophyll content (micromole per square meter) was calculated from a standard curve, chlorophyll (micromole per square meter)=$10^{(M^{\wedge}\ 0.261)}$, where M is the chlorophyll meter reading (Markwell et al. 1995).

Chemicals

All reagents and solvents used in the study were purchased from Fisher Scientific (Pittsburgh, PA, USA) or Sigma-Aldrich (St Louis, MO, USA).

Table 1 Survival and development (mean ± standard error) of *S. frugiperda* larvae after feeding on three corn inbreds for 1 week under greenhouse conditions

Corn germplasm	Survival rate (%)	Larval biomass[b] (g)	Pupal biomass (g)	Developmental time[c] (days)
Ab24E[a]	53.3±6.7a	0.05±0.007a	0.24±0.006a	15.7±0.16b
Mp708[a]	26.7±6.7b	0.02±0.003b	0.26±0.016a	17.2±0.60a
FAW7050[a]	33.3±6.7b	0.02±0.002b	0.25±0.014a	17.6±0.19a

Recovered larvae were reared individually on modified Pinto bean diet. Means followed by different lowercase letters within a column denote significant difference at $\alpha=0.05$

[a] $N=6$ for Ab24E and FAW7050 and $N=3$ for Mp708

[b] Larval biomass at the time of recovery

[c] Time from onset of experiment to pupation in days

Statistical Analyses

S. frugiperda recovery rate, larval and pupal biomass, and the development from onset of experiments to pupation were analyzed with one-way analysis of variance (ANOVA). Recovery rate was arcsin square-root transformed prior to analysis. There were originally six replicates for variety Ab24E and FAW7050 and three replicates for Mp708. Because the only *S. frugiperda* larva recovered in one replicate from variety FAW7050 was accidentally killed, there were only five replicates included in the analyses for larval and pupal biomass and development. Damage rating in the field was analyzed with nonparametric Kruskal–Wallis tests. Photosynthesis data were analyzed by a 3 (corn germplasm)×2 (*S. frugiperda* infested and uninfested) ANOVA. Analyses were separately conducted at different concentrations of CO_2 and light intensity. Total soluble protein, amino acids, glucose, TNC, P/C ratio, peroxidase, and LOX3 activity were analyzed by a 3 (corn inbred)×2 (*S. frugiperda*-infested and uninfested) ANOVA. P/C ratio was square-root transformed before analysis. If the null hypothesis of the overall model was rejected at $\alpha=0.05$, means were further separated by least significant difference or Students' *t* test. All statistical analyses were conducted using the SAS software package (SAS Institute 1999).

Results

Development and Damage of *S. frugiperda* on Corn Germplasm Lines

Development of *S. frugiperda* in the greenhouse trial (Table 1) and damage in the field trial (Table 2) indicated that variety Ab24E was susceptible, while Mp708 and FAW7050 were resistant. More larvae were recovered from variety Ab24E than Mp708 and FAW7050 ($F=3.88$, $df=2, 12$, $P<0.05$). Biomass of *S. frugiperda* larvae feeding on Ab24E for 7 days was significantly greater than those feeding on Mp708 and FAW7050 for the same period of time ($F=14.66$, $df=2, 12$, $P<0.001$). Larvae of *S. frugiperda* took 8–11% less time to develop to the pupal stage after feeding on Ab24E for 7 days than on the other two germplasm lines ($F=16.34$, $df=2, 11$, $P<0.001$). Corn germplasm lines did not affect pupal weight ($F=0.89$, $df=2, 11$, $P=0.44$).

Table 2 Damage by *S. frugiperda* larvae and leaf chlorophyll content (mean ± standard error) of three corn germplasms under the field conditions

Corn germplasm	Damage rating[b]		Leaf chlorophyll (μmol m^{-2})
	7 DAI	14 DAI	
Ab24E[a]	5.0±0.71°	8.8±0.25a	523.07±11.49b
Mp708[a]	2.8±0.48b	3.8±0.25b	522.56±11.78b
FAW7050[a]	2.3±0.48b	4.0±0.91b	610.37±10.84a

Means followed by different lowercase letters within a column denote significant difference at $\alpha=0.05$

DAI days after *S. frugiperda* infestation

[a] $N=4$ for all three germplasm lines

[b] According to Davis et al. (1992) and Smith et al. (1994), with 1=no damage or few pinholes, 2=few short holes on several leaves, 3=short holes on several leaves, 4=several leaves with short holes and a few long lesions, 5=several holes with long lesions, 6=several leaves with lesions <2.5 cm, 7=long lesions common on one half of the leaves, 8=long lesions common on one half to two thirds of leaves, and 9=most leaves with long lesions

Table 3 Protein, amino acid, and nonstructural carbohydrate (mean ± standard error) of three corn germplasm lines

Corn germplasm	Total protein content (mg g^{-1} FW)		Amino acids (μmol g^{-1} FW)	Glucose (μmol g^{-1} FW)		TNC μmol g^{-1} FW)[e]	P/C (*w*/*w*)
	Control	Damaged		Control	Damaged		
Ab24E[a]	10.06±0.63b	11.12±1.31a	6.43±0.88a	6.39±0.78a	9.19±1.20a	24.25±2.51a	2.69±0.28b
Mp708[a]	5.74±0.50a	9.41±0.93a[b]	9.48±0.64b	8.58±0.94a[c]	15.59±0.72b	33.23±3.20a	1.27±0.12a
FAW7050[a]	8.97±0.60b	11.97±0.62a[c]	11.00±1.16b	8.45±0.72a[d]	17.26±1.68b	27.94±3.78a	2.29±0.20b

Means followed by different lowercase letters within a column denote significant difference at α=0.05

FW fresh weight of leaf tissue, *P/C* protein to TNC ratio

[a] N=6 for Ab24E and FAW7050 and N=3 for Mp708

[b] Significant difference between control and damaged treatments of the same corn germplasm at α=0.05

[c] Significant difference between control and damaged treatments of the same corn germplasm at α=0.01

[d] Significant difference between control and damaged treatments of the same corn germplasm at α=0.001

[e] Total nonstructural carbohydrate as sum of glucose and starch (equivalents of glucose)

S. frugiperda injury in the field was significantly affected by corn germplasm lines (7 days after *S. frugiperda* infestation (DAI): X^2=7.03, *df*=2, P<0.05; 14 DAI: X^2=7.63, *df*=2, P<0.05). Damage on Ab24E was consistently greater than damage on Mp708 and FAW7050 (Table 2).

Influence of *S. frugiperda* on Nutritional Properties of Germplasm Lines

The data of protein, amino acid, and nonstructural carbohydrates are summarized in Table 3. Ab24E and FAW7050 had greater constitutive foliar total soluble protein than Mp708 (F=9.42, *df*=2, 12, P<0.01). *S. frugiperda* injury increased total protein content in Mp708 and FAW7050 (F=12.04, *df*=1, 4, P<0.05 for Mp708; F=12.05, *df*=1, 10, P<0.01 for FAW7050) compared with the control plants. Amino acid levels in FAW7050 and Mp708 were greater than Ab24E (F=5.91, *df*=2, 27, P<0.01). Constitutive glucose, starch, and TNC contents did not differ among the three germplasm lines (P>0.05). However, Mp708 and FAW7050 had over 24% higher constitutive glucose than Ab24E. Additionally, *S. frugiperda* damaged leaves of Mp708 and FAW7050 had greater glucose levels than Ab24E (F=9.54, *df*=2, 12, P<0.01). P/C ratios of all three corn lines were greater than 1:1 and were not affected by *S. frugiperda* feeding (F=0.02, *df*=1, 22, P>0.05; Table 3). P/C ratio of Ab24E and FAW7050 was greater than those of Mp708 (F=6.27, *df*=2, 22, P<0.05).

Effect of *S. frugiperda* Infestation on Peroxidase and LOX3 Activities

Constitutive peroxidase activity of Mp708 was higher than Ab24E and FAW7050 (F=10.24, *df*=2, 12, P<0.01; Fig. 1). *S. frugiperda* damage increased peroxidase activity of Ab24E (F=7.19, *df*=1, 10, P<0.05). No significant difference in peroxidase activity was observed among the damaged leaf tissue of the three germplasm lines (F=0.56, *df*=2, 12, P=0.58). *S. frugiperda* damage on leaves decreased LOX3 activity of Mp708 (data not shown; F=18.45, *df*=1, 4, P<0.05). No significant difference in LOX3 activity was detected among germplasm lines.

Photosynthetic Rates of the Corn Germplasm Lines

Because none of the interactions between *S. frugiperda* infestation and germplasm line was significant (all P>0.05), further analysis was performed to examine main effects. Corn germplasm lines significantly (F=7.41, *df*=2, 19, P<0.01) affected photosynthetic rate in the greenhouse, and the rate of *S. frugiperda*-resistant Mp708 was greater

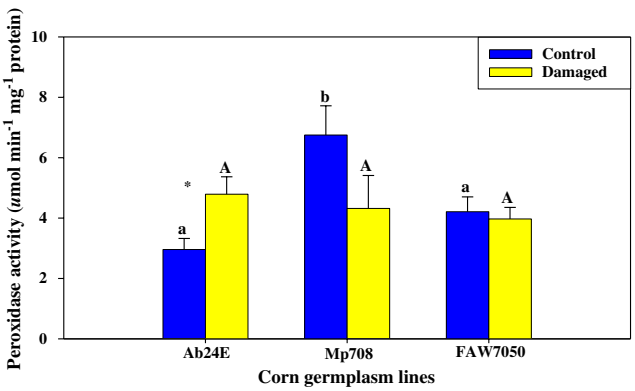

Fig. 1 Peroxidase activity (micromole per minute per milligram protein) of three corn germplasm lines. *Different lowercase letters* (*a–b*) *above bars* denote significant differences among corn germplasm lines of the undamaged (control) plants at α=0.05, whereas *capital letter* (*A*) denotes that no significant difference was detected among the *S. frugiperda*-damaged leaves. *α=0.05 denotes a significant difference between control and *S. frugiperda*-damaged plants of the same germplasm line. N=6 for Ab24E and FAW7050 and N=3 for Mp708

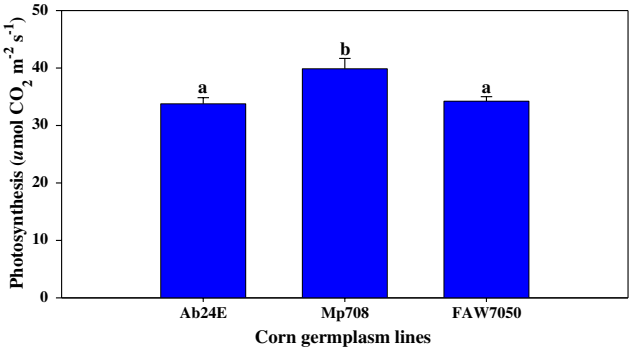

Fig. 2 Photosynthetic rate (micromole CO_2 per square meter per second) of three corn germplasm lines. *Different lowercase letters above bars* denote significant difference at α=0.05. N=6 for Ab24E and FAW7050 and N=3 for Mp708

than either of the other two lines (Fig. 2). However, *S. frugiperda* infestation did not affect photosynthesis using survey measurements (F=0.19, df=1, 19, P=0.67), nor A/Ci curve at each CO_2 concentration (all P>0.05), nor light curve at either of eight light intensities (all P>0.05). Corn germplasm lines had no effects on photosynthetic capacity at any of the light intensities (all P>0.05) under greenhouse conditions. Photosynthetic rates of Mp708 and FAW7050 at CO_2 concentrations of 300, 400, 600, and 800 ppm was greater than that of Ab24E (300 ppm: F=4.28, df=2, 15, P<0.05; 400 ppm: F=8.37, df=2, 51, P<0.001; 600 ppm: F=6.82, df=2, 15, P<0.01; 800 ppm: F=7.26, df=2, 15, P<0.01; Fig. 3). Mp708 and FAW7050 photosynthetic rates were significantly greater than Ab24E when CO_2 levels were greater than 300 ppm.

Leaf chlorophyll of FAW7050 was greater than that of Ab24E and Mp708 (F=20.40, df=2, 90, P<0.001; Table 2), but leaf chlorophyll content was not affected by *S. frugiperda* infestation (F=2.51, df=1, 90, P>0.05). The interaction between *S. frugiperda* infestation and corn germplasm was not significant (F=0.09, df=2, 90, P=0.92).

Discussion

Plant nutritional levels and allelochemicals often affect plant suitability and resistance to herbivory. Amino acids and proteins are two major sources of nitrogen for phytophagous insects, and herbivores provided with added proteins or nitrogen typically develop faster and survive and reproduce better (Mattson 1980; Woods 1999; Lee et al. 2002). Correspondingly, many herbivores have evolved various strategies that maximize the acquisition of amino acids or proteins (White 1993).

FAW7050 and Mp708 had higher levels of amino acids than susceptible Ab24E in the study. *S. frugiperda* development in the greenhouse and damage ratings in the field confirmed that Ab24E was susceptible while FAW7050 and Mp708 were resistant. If amino acid amount were the only determinant in the development of *S. frugiperda*, then larvae growth on FAW7050 and Mp708 would be faster, which is contrary to what was observed. Nutrient balance, in particular, P/C ratio has been suggested as important for development and preference of many insects in the laboratory (Simpson and Raubenheimer 1993; Lee et al. 2002; Bede et al. 2007), and most insects studied, particularly generalist insects, selected artificial diets with a P/C>1 over those with a P/C<1 (Bede et al. 2007). P/C of all three corn germplasm lines in this study were greater than 1:1, and P/C in susceptible Ab24E and resistant FAW7050 were higher than in resistant Mp708. The faster

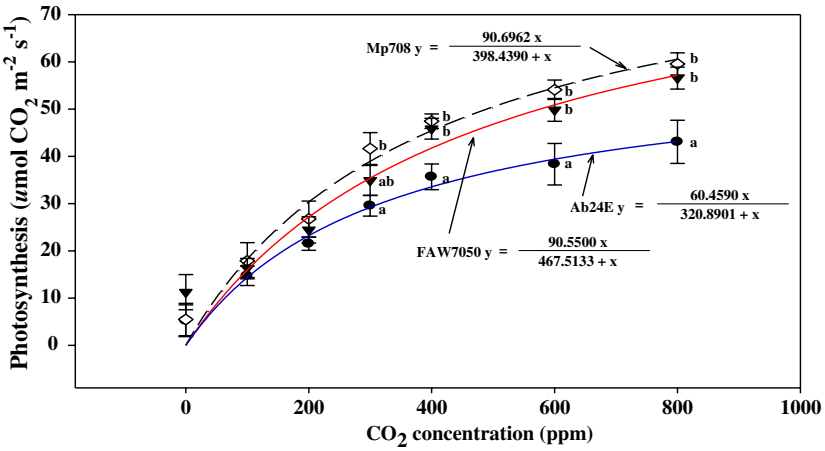

Fig. 3 Photosynthetic rate (micromole CO_2 per square meter per second) of three corn germplasm lines at various CO_2 concentrations using the combined data (N=6 for all CO_2 levels, except 400 ppm with N=18 using the automated program for A/Ci curve). *Different lowercase letters* next to data points of the same CO_2 concentration denote significant difference at α=0.05. The regression equation of photosynthetic rate against CO_2 concentration was nonlinear (*hyperbola): y=ax/(b+x). Regression coefficient (r) values for varieties Ab24E (with filled circle), Mp708 (with empty diamond), and FAW7050 (with filled triangle) were 0.99, 0.99, and 0.98, respectively*

development of *S. frugiperda* in Ab24E and slower growth in Mp708 was consistent with the higher P/C in Ab24E and lower P/C in Mp708, respectively. The slower growth of *S. frugiperda* in FAW7050 that had equally high P/C with Ab24E cannot be explained solely by P/C.

Proteins are another main source of nitrogen, and soluble proteins in plants can be broadly grouped into defensive (e.g., proteinase inhibitors) and nondefensive. Soluble proteins of all three corn inbred lines accounted for less than 1% of total leaf fresh weight, suggesting that arthropods in nature are faced with a shortage of nitrogen (Mattson 1980; White 1993). Susceptible Ab24E had higher constitutive protein levels than the resistant FAW7050 and Mp708, while no difference in total protein content among damaged corn lines was observed. This was because *S. frugiperda* feeding induced protein production in FAW7050 and Mp708, but had little effect in Ab24E. Herbivory induces plant defensive chemicals such as protease inhibitors (Jongsma et al. 1994; Stout et al. 1994, 1998; Lawrence and Koundal 2002), and many of these are detrimental to herbivore fitness (Broadway and Duffey 1988; Ryan 1990; Stout et al. 1998; Leo et al. 2001; Ussuf et al. 2001; Lawrence and Koundal 2002; Zavala et al. 2004). Therefore, the lower survival and slowed development of *S. frugiperda* in resistant FAW7050 and Mp708 might be attributable to elevated plant defensive compounds following herbivory in these two germplasm lines and to a lower P/C in Mp708. The faster growth in susceptible Ab24E was due mainly to higher amounts of constitutive nondefensive proteins and little induction of defensive compounds.

Although many plant oxidative enzymes such as peroxidase and LOX3 are involved in host plant resistance by decreasing the nutritive value of host plants (Shukle and Murdock 1983; Felton et al. 1994; Duffey and Stout 1996; Ni et al. 2001; Ni and Quisenberry 2003), neither peroxidase nor LOX3 was responsible for the lower survival and protracted development of *S. frugiperda* in this study. First, resistant Mp708 had greater peroxidase activity than susceptible Ab24E and resistant FAW7050, and no significant difference in peroxidase activity among damaged corn inbred lines was observed. Second, no significant difference in LOX3 activity among damaged corn germplasm lines was observed. However, the high inducibility of peroxidase might be a good indicator of plant susceptibility as observed in pecan, *Carya illinoinensis* (Wangenh.) K. Koch (Juglandaceae; Chen et al., unpublished data).

Photosynthesis is a complex, integrated, and dynamic process that is regulated by both source (e.g., light and CO_2) and sink (e.g., photosynthates, such as starch; Paul 1981; Paul and Foyer 2001). Because starch levels were not affected in germplasm lines (data not shown) and because light and CO_2 were controlled, the greater photosynthetic rates in resistant Mp708 and FAW7050 (Fig. 3) compared to Ab24E may be intrinsic. The greater rates in FAW7050 and Mp708 were likely due to greater light-independent reactions that assimilate CO_2 and produce carbohydrates. Such difference, in turn, resulted in higher (over 24%) glucose content in Mp708 and FAW7050 than that in Ab24E. Greater photosynthetic rates also led to higher levels of amino acids in these lines. *S. frugiperda* feeding injury led to nearly a twofold increase of glucose levels in Mp708 leaves and more than a twofold increase in FAW7050, compared to their corresponding undamaged controls. However, starch content was not affected. This suggests that *S. frugiperda* feeding damage might have decreased the conversion of glucose to some compounds in these lines, but not to total protein content, as total protein content was elevated in the *S frugiperda*-damaged plants of these lines.

In summary, the three corn germplasm lines differed in their mechanisms of resistance to *S. frugiperda*, and P/C and induced plant defensive compounds (e.g., protease inhibitors) need to be considered to explain resistance mechanisms. Elevated protein amounts in resistant germplasm lines Mp708 and FAW7050 following *S. frugiperda* injury was probably due to greater conversion of photosynthates to defensive compounds.

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Disease Status and Population Origin Effects on Floral Scent: Potential Consequences for Oviposition and Fruit Predation in A Complex Interaction Between A Plant, Fungus, and Noctuid Moth

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Abstract In the *Silene latifolia–Hadena bicruris* nursery pollination system, the *Hadena* moth is both pollinator and seed predator of its host plant. Floral scent, which differs among *S. latifolia* individuals and populations, is important for adult *Hadena* to locate its host. However, the success of moth larvae is strongly reduced if hosts are infected by the anther smut fungus *Microbotryum violaceum*, a pathogen that is transmitted by flower visitors. There were no qualitative differences between the scent of flowers from healthy and diseased plants. In addition, electroantennographic measurements showed that *Hadena* responded to the same subset of 19 compounds in samples collected from healthy and diseased plants. However, there were significant quantitative differences in scent profiles. Flowers from diseased plants emitted both a lower absolute amount of floral scent and had a different scent pattern, mainly due to their lower absolute amount of lilac aldehyde, whereas their amount of (*E*)-β-ocimene was similar to that in healthy flowers. Dual choice behavioral wind tunnel tests using differently scented flowers confirmed that moths respond to both qualitative and quantitative aspects of floral scent, suggesting that they could use differences in floral scent between healthy and infected plants to discriminate against diseased plants. Population mean fruit predation rates significantly increased with population mean levels of the emission rates of lilac aldehyde per flower, indicating that selection on floral scent compounds may not only be driven by effects on pollinator attraction but also by effects on fruit predation. However, variation in mean emission rates of scent compounds per flower generally could not explain the higher fruit predation in populations originating from the introduced North American range compared to populations native to Europe.

Keywords Anther smut fungus · *Microbotryum violaceum* · *Hadena bicruris* · Lilac aldehyde · Nursery pollination · *Silene latifolia* · Flower scent · Dynamic headspace · Wind tunnel biotest · Electroantennographic measurements

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Introduction

In many herbivorous insects, preadult stages have little opportunity to move away from their oviposition site; hence, the mother insect is effectively choosing the diet of her offspring (Mayhew 1997). The oviposition preference–offspring performance hypothesis predicts that oviposition preference should correlate with host suitability for offspring development (Jaenike 1978; Scheirs et al. 2000), as ovipositing on high quality hosts will increase fitness (Awmack and Leather 2002). However, empirical studies have not unequivocally supported this hypothesis (Thompson and Pellmyr 1991; Mayhew 1997, 2001). The quality of a

host plant for herbivores may not only be influenced by the plant itself but also by third parties such as fungi that use the same host plant(s) as the herbivores (Hatcher 1995). Although infection of plants by fungi can have neutral or positive effects on host-plant quality (summarized in Hatcher 1995), it more often has a negative effect on herbivore performance (e.g., Simon and Hilker 2003; Laine 2004). Research on the effects of plant infection by pathogenic fungi on herbivore performance has focused mainly on vegetative plant tissues, whereas little is known about the effects of plant pathogenic fungi on insects that feed on reproductive tissues.

Recently, the anther smut fungus *Microbotryum violaceum* was shown to strongly affect the interaction between *Silene latifolia* and the noctuid moth *Hadena bicruris* (Biere et al. 2002). *Hadena* is not only a pollinator of this species but also lays its eggs within the flowers, where the larvae subsequently feed on developing seeds. This system is one of 14 known nursery pollination systems (Dufaÿ and Anstett 2003; Kato et al. 2003), including the well-studied yucca/yucca moth and fig/fig wasps relationships. *Microbotryum violaceum* sterilizes the plant and produces its spores in the anthers of infected plants, which are transmitted by *Hadena* and other flower visitors. Besides this direct effect on the plant, the fungus may reduce the pollination effectiveness of *Hadena*, which transmits not only pollen but also fungal spores. This negatively affects pollen germination (Marr 1998) and results in infected plants producing sterile flowers.

Infection also affects *Hadena*. First, *Hadena* develops less successfully on diseased than on healthy plants because diseased plant flowers do not produce seeds, and larvae are forced to feed on tissues with lower nutritional quality, resulting in smaller size and lower survival (Biere et al. 2002). Second, larvae on diseased plants suffer a higher predation risk from Hymenopteran parasitoids. This is because the fruit of healthy plants provides protection from parasitoid attack during the stages that caterpillars are concealed within the developing fruit. In diseased plants, ovary development is arrested at an early stage, and caterpillars are not able to gain protection from concealment within fruits (Biere et al. 2002). In natural populations, such larval parasitism may account for 40% of *Hadena* mortality (Elzinga et al. 2007). According to the oviposition preference–offspring performance hypothesis, it can be expected that female moths avoid depositing eggs in diseased flowers, and indeed, oviposition rates have been found to be much higher on flowers of healthy than diseased plants (Biere and Honders 2006). However, the exact cues used by *Hadena* for discriminating between healthy and diseased plants are unknown (Biere and Honders 2006). One of these cues may be floral scent, given that *Hadena* is attracted to flowers by their scent, whereas lilac aldehyde, the most typical compound of *S. latifolia* flowers, has been identified as the most behaviorally attractive compound (Dötterl et al. 2006).

These findings raise the question of whether there is a difference in scent between anther-smut infected and uninfected flowers that could be used by *Hadena* to discriminate between them. Qualitative (presence and absence of compounds), quantitative (absolute amount of single compounds or total scent), and/or semiquantitative (relative/percentage amount of compounds in relation to total amount) differences in scent between pathogen-infected and uninfected plants have been well documented for vegetative plant parts (e.g., Schütz et al. 1999; Cardoza et al. 2002; Rostás et al. 2006; Johne et al. 2008) including "pseudo flowers," which are fungal and fungus-induced vegetative plant structures that mimic flowers by visual floral mimicry, production of nectar rewards, and/or floral fragrances (Roy and Raguso 1997; Raguso and Roy 1998; Kaiser 2006). For vegetative tissue and "pseudo flowers," the most prominent differences in scent have been documented for plants infected by fungi that depend on insects for spore transmission (Roy and Raguso 1997; Raguso and Roy 1998; Naef et al. 2002; Kaiser 2006; Schiestl et al. 2006). In some cases, the changes in scent pattern due to infection have resulted in behavioral changes in phytophagous insects or spore transmitters (e.g., Roy and Raguso 1997; Cardoza et al. 2003; Schiestl et al. 2006; Johne et al. 2008). However, we are not aware of any study that shows the effects of fungal infection on the floral scent of true flowers.

Recently, large qualitative as well as (semi)quantitative differences in floral scent were described among healthy *S. latifolia* populations, i.e., independent of disease status of the plants (Dötterl et al. 2005b, 2007). Furthermore, differences in fruit predation rates by *H. bicruris* among *S. latifolia* populations were observed in a common garden experiment (Wolfe et al. 2004). These findings suggest that population-level fruit predation rates could be explained by among-population variation in floral scent, which could be determined by analyzing population mean fruit predation rates as a function of population mean emission rates of floral scents to which *Hadena* has shown significant antennal responses.

The two main goals in this research were to test whether there is a difference in scent between uninfected and anther-smut-infected flowers that could be used by *Hadena* for discriminating between them and whether differences in fruit predation rates by *H. bicruris* among *S. latifolia* populations could be explained by among-population variation in floral scent. The specific questions are the following: (1) Are there differences in floral scent between flowers of healthy and diseased plants of *S. latifolia*? (2) If the scent differs, is *H. bicruris* able to detect these

differences? (3) Is attraction of the moth affected by scent quantity and composition? (4) Can differences in predation rates among populations be partly explained by differences in floral scent? To answer the first two questions, we compared the volatile profiles of flowers from healthy and diseased plants from a number of populations and measured antennal responses of *H. bicruris* to the emitted compounds. We performed dual-choice behavioral tests on *H. bicruris* to differently scented flowers to address the third question. To answer the fourth question, we tested whether the population mean predation rates by *H. bicruris* measured by Wolfe et al. (2004) on *S. latifolia* plants originating from different North American and European populations that were grown in a common garden are associated with the population specific floral scent spectra emitted by these plants (see Dötterl et al. 2005b, 2007).

Methods and Materials

Plant Material and Fungal Inoculation For experiments on scent differences between healthy and diseased flowers, we used plants from seven different *S. latifolia* populations, two originating from Europe and five from North America (Fig. 1, see also Wolfe et al. 2007). To compare scent profiles of healthy and diseased flowers within populations, we created full-sib families within populations by making crosses between two randomly chosen parents from each population. Twelve seeds of each cross were assigned to a control treatment and 12 to a fungal inoculation treatment group. The 12 seeds from each cross and treatment were germinated by placing them in a 9-cm Petri dish on moist filter paper in a growth cabinet under 16 h 25°C L/8 h 15°C D after inbibing in the dark for 5 d at 4°C. Plants and treatments were part of a larger experiment that addressed the susceptibility of European and North American plants to inoculation (Biere and Verheggen, unpublished data).

Inoculum of *M. violaceum* (Pers.) G. Deml & Oberwinkler was prepared as follows. Diploid teliospore samples were collected from flowers of infected *S. latifolia* plants at Fyfield, Hampshire, UK (51°13′06 N, 1°34′46 E). Spores from individual flowers were plated onto yeast–glucose–agar (YGA) and incubated at 23°C for 5 days. Single spore colonies, containing the haploid meiotic products (sporidia) of both mating types, a1 and a2, were transferred to new YGA plates and incubated as above. The resulting single sporidia colonies were isolated and cultured as above for multiplication. Sporidia of opposite mating type (a1 and a2) can conjugate to form infectious hyphae. The mating type of isolated single sporidia colonies was determined by mixing them with reference sporidia of known mating type on water agar and checking for conjugations after incubation overnight at 15°C. Two sporidial isolates of opposite mating type that originated from different plants were used for inoculation. Inoculation suspensions were made by mixing 10^7 sporidia of each mating type in sterile demineralized water overnight in a rotary shaker at 15°C.

Three days after *S. latifolia* seeds started to germinate, those assigned to the inoculation treatment were inoculated by pipetting 2 ml of the inoculum suspension into a Petri dish, while those assigned to the control treatment received 2 ml of sterile demineralized water (mock inoculation). To increase infection rates in the inoculation treatments, this procedure was repeated 10 days later for each Petri dish using fresh inoculum or control treatments. After 16 days, plant seedlings were transplanted into individual plastic container pots (5-cm diameter and 25-cm depth) containing potting soil and reared in a greenhouse (16 hr L, 21±2°C throughout) for 15 weeks until they were transplanted to an experimental garden at The Netherlands Institute of Ecology (Heteren), where flowering started on May 19, 2005. Successful inoculations are easily assessed by visual inspection of the presence of purple-brown spores instead of creamy pollen in the anthers of male as well as female plants (the latter undergo a fungus-induced morphological sex change).

Insects and Behavioral Tests A *H. bicruris* culture was established by collecting eggs laid in *S. latifolia* flowers in the surroundings of Bayreuth (Germany). Half of the larvae

Fig. 1 Geographic origins of the five North American and two European populations of *Silene latifolia* analyzed. The *digits within the symbols* indicate the number of floral scent samples collected in order to test for differences in scent between healthy plants (*open symbols*) and plants infected by *Microbotryum violaceum* (*solid symbols*)

were reared on freshly collected fruits of *S. latifolia* during all five larval stages, and the others were fed with artificial diet as described by Dötterl et al. (2006). Adults were provided with a sugar solution (30%, same amounts of fructose and glucose corresponding to *S. latifolia* nectar as described by Witt et al. 1999). Two- to 7-day-old flower-inexperienced female and male moths were used for the experiments.

Behavioral tests were conducted in a 160-×75-×75-cm wind tunnel, the same as described by Dötterl et al. (2006). A Fischbach speed controller fan (D340/E1, FDR32, Neunkirchen, Germany) pushed air through the tunnel with an air speed of 0.35 m s^{-1}. Four charcoal filters (145× 457 mm; carbon thickness, 16 mm; Camfil Farr) cleaned the incoming air.

Two dual-choice assays were used to obtain information on the importance of both the total amount of scent and composition of the scent for attracting *Hadena*. In one assay, we compared the attractiveness of two flowers emitting a similar scent (qualitatively and semiquantitatively) but differing in their total amount of scent. In the other test, we compared two flowers that differed in both their quantity of emitted scent and their semiquantitative scent pattern. The flowers chosen represented the most typical chemotypes found in *S. latifolia* (lilac aldehyde vs. phenylacetaldehyde dominated) and the typical quantitative range of scent emission found in this plant (Dötterl et al. 2005b). To eliminate visual stimuli, flowers were presented at the upwind end of the tunnel behind gauze and different metal grids, so that they were invisible to the moths. Single moths were released at the downwind end of the tunnel, and their behavior was observed for at most 5 min. Data were recorded by personal observation for moths flying 'zigzag' to within 5 cm of one of the flowers. Three and four out of 21 individuals used in these two tests, respectively, did not respond at all and were not used for analyses (see Results). The experiments were performed at night with red light illumination (<0.01 μE) 1–3 h after the start of the dark period. The temperature was adjusted to 22–24°C, but the relative humidity was left unchanged.

Volatile Collection Floral scent was collected for three separate purposes: (1) to compare scents between infected and uninfected flowers, (2) to determine the scent of flowers used in behavioral assays, and (3) to obtain scent samples for electrophysiological measurements.

To compare scents between infected and uninfected flowers, we collected floral scent on June 11, 2005 (10–12 P.M.) from 12 healthy and 14 diseased female plants (two to six plants per cross, see Fig. 1, and one flower per plant) by dynamic headspace as described by Dötterl et al. (2005b). One flower per plant was picked, enclosed within a polyester oven bag (10×8 cm; Toppits®, Germany), and 1 min later, scent collection was started. In *S. latifolia*, the volatile emission from cut flowers does not differ from that of flowers still attached to the plant if scent is collected immediately after flower picking (Dötterl, unpublished data).

To determine the composition and amount of scent emitted by the single flowers used in the behavioral tests, each picked flower was sampled two times: immediately after picking the flowers before conducting the biotests and also after the biotests (about 2 h later). Results of these two measurements were used to calculate the mean amount and composition of emitted volatiles during the tests.

In both experiments (infected–uninfected; behavioral assay), volatiles were trapped for 2 min in an adsorbent tube by the use of a membrane pump (G12/01 EB, ASF Thomas, Inc.) with a flow rate of 200 ml min^{-1}. The adsorbent tube was filled with a mixture of 1.5 mg Tenax-TA (mesh 60–80) and 1.5 mg Carbotrap (mesh 20–40). Only young flowers, mostly in their first night of opening, were used for scent collection to avoid confounding effects of ontogenetic changes, e.g., following pollination (Dötterl et al. 2005b; Mühlemann et al. 2006). To distinguish between plant volatiles and ambient contaminants, surrounding air was collected for comparison.

The scent samples used for electrophysiological analyses were six in total and were collected from a subset of the plants used to study effects of fungal infection on floral scent profiles. Four samples were collected from a full-sib cross that originated from one of the European populations (two healthy and two diseased plants), and two samples were collected from a full-sib cross that originated from one of the North American populations (one healthy and one diseased plant). To obtain the samples, a different dynamic headspace method was used. For each sample, floral scent was collected from ten to 40 flowers (the night of June 11 to the morning of June 12). The flowers were enclosed in situ in the same oven bags as used above, but the bag size was larger (30×30 cm). The emitted volatiles were trapped in large adsorbent tubes filled with 15 mg of Tenax-TA 60–80 and 15 mg of Carbotrap 20–40. Volatiles were eluted with 70 μl of acetone (SupraSolv, Merck KgaA, Germany) and stored in a freezer at −80°C for later use in the electrophysiological analyses (see below).

GC-MS Analyses Floral scent samples from the inoculation and behavioral experiments were analyzed on a Varian Saturn 3800 gas chromatograph (GC) fitted with a 1079 injector and a ZB-5 column (5% phenyl polysiloxane; length, 60 m; inner diameter, 0.25 mm; film thickness, 0.25 μm; Phenomenex) and a Varian Saturn 2000 mass spectrometer (MS). The adsorbent tubes were inserted via Varians Chromatoprobe into the GC injector (Amirav and Dagan 1997). The injector split vent was opened (1/20), and the injector was heated at 40°C to flush any air from the system. After 2 min, the split vent

was closed and the injector heated at 200°C min^{-1}, then held at 200°C for 4.2 min, after which the split vent was opened (1/20) and the injector cooled down. Electronic flow control was used to maintain a constant helium carrier gas flow rate (1.8 ml min^{-1}). The GC oven temperature was held for 7 min at 40°C, then increased by 6°C min^{-1} to 260°C, and held for 1 min at this temperature. Mass spectra were taken at 70 eV with a scanning speed of one scan per second from *m/z* 30 to 350.

Analysis of the data was performed using the Saturn Software package 5.2.1. To identify the floral scent compounds of the GC-MS spectra, the NIST 02 and MassFinder 3 databases were used, and identifications were confirmed by comparison of retention times with published data (Adams 1995). Identification of some compounds was also confirmed by comparison of mass spectra and retention times with those of authentic standards. Volatiles emitted from vegetative plant parts were excluded for the analyses. To quantify the amount of each volatile in the blend, known amounts of monoterpenoids, benzenoids, and fatty acid derivatives were injected, and the mean peak area of these compounds was used for quantification.

Electrophysiological Analyses To test whether the antennae of *H. bicruris* can detect (respond to) different compounds present in flowers of healthy and diseased plant individuals, we performed electrophysiological analyses, gas chromatography coupled to electroantennographic detection (GC-EAD). The six scent samples collected for these analyses were tested on four antennae of three different moth individuals; a total of 14 runs were conducted. Each antenna was tested at least on one healthy and one diseased sample of a specific population. Insects were reared from eggs as described above.

Analyses were performed with the GC-EAD system described by Dötterl et al. (2005a). The GC-EAD system consisted of a gas chromatograph (Vega 6000 Series 2, Carlo Erba, Rodano, Italy) equipped with a flame ionization detector (FID) and an EAD setup (heated transfer line, two-channel USB acquisition controller) provided by Syntech (Hilversum, The Netherlands). A volume of 1 µl of an acetone–floral scent sample was injected splitless at 60°C, followed by opening the split vent after 1 min and heating the oven at a rate of 10°C min^{-1} to 200°C. The end temperature was held for 5 min. A ZB-5 column was used for analyses (length, 30 m; inner diameter, 0.32 mm; film thickness, 0.25 µm; Phenomenex). The column was split at the end by the four arm flow splitter GRAPHPACK 3D/2 (Gerstel, Mülheim, Germany) into two pieces of deactivated capillary (length, 50 cm; inner diameter, 0.32 mm) leading to the FID and EAD setup. Makeup gas (He, 16 ml min^{-1}) was introduced through the fourth arm of the splitter. For the EAD, both sides of an excised antenna were plugged into glass micropipette electrodes filled with insect ringer solution (8.0 g l^{-1} NaCl, 0.4 g l^{-1} KCl, 4 g l^{-1} $CaCl_2$), respectively, and connected to silver wires.

To identify the structure of the compounds eliciting signals in the insect antennae, 1 µl of the acetone–floral scent samples was placed into a quartz vial in the injector port of the GC by means of the ChromatoProbe and then analyzed by GC-MS as described above.

Seed Predation in Relation to Floral Scent in Healthy Plants To test whether differences in floral scent profiles between populations were associated with differences in fruit predation by *H. bicruris*, we calculated mean predation rates for female plants from nine European and 12 North American populations using data (collected in 2002) from a previous study performed in the same common garden at The Netherlands Institute of Ecology, Heteren (Wolfe et al. 2004). We also used the mean relative amount of the subset of floral scent compounds to which *H. bicuris* showed a significant response in the electrophysiological analyses (GC-EAD; Dötterl et al. 2006 and the present study) for the same 21 populations but calculated using floral scent (collected in 2003) from a previously published study (Dötterl et al. 2005b). The compounds used were the following: 3-methylbutylaldoxime, (*Z*)-3-hexen-1-ol, benzaldehyde, myrcene, (*Z*)-3-hexenyl acetate, benzylalcohol, (*E*)-ß-ocimene, phenylacetaldehyde, guaiacol, methyl benzoate, 2-phenylethanol, veratrole, lilac aldehyde, benzyl acetate, methyl salicylate, lilac alcohol, benzenepropanol, (*E*)-cinnamaldehyde, (*E*)-cinnamyl alcohol, (*E*)-cinnamyl alcohol acetate, and benzyl benzoate.

Statistical Analyses To test for a difference in the total amount of scent emitted by flowers from healthy and diseased plants, a Mann–Whitney *U* Test was performed using the STATISTICA 7.1 package (StatSoft Inc. 2004).

For detailed analyses of qualitative and quantitative differences in scent composition among samples, we performed analyses of similarities (ANOSIM) using the program package Primer 6.1.6 (Clarke and Warwick 2001; Clarke and Gorley 2006). ANOSIM is a commonly used multivariate procedure roughly analogous to analysis of variance (ANOVA)/multivariate ANOVA that operates directly on a (dis)similarity matrix. It yields a test statistic *R* that is a relative measure of separation between a priori defined groups, based on differences of mean ranks between and within groups. An *R* value of "0" indicates a completely random grouping, whereas a value of "1" indicates that samples within groups are more similar to each other than to any sample from a different group (Clarke and Gorley 2006). Statistical significance of *R* is assessed by random permutations of the grouping vector to obtain an empirical distribution of *R* under the null model.

For analyses of qualitative differences in scent emission, we calculated the qualitative Sørensen index using Primer 6.1.6 to determine pairwise qualitative similarities among samples. Based on this similarity matrix, we performed a two-level nested ANOSIM [factors population and disease status (healthy or diseased) nested within population] to test for qualitative differences in scent between healthy and diseased plants.

For analyses of quantitative differences in scent emission, we calculated the Bray–Curtis quantitative similarity index using Primer 6.1.6 to assess pairwise quantitative similarities between the different samples based on the total amount emitted per compound. Non-metric multidimensional scaling was used (based on the Bray–Curtis similarities) in Primer to ordinate the scent samples of the 12 healthy and 14 diseased samples of the different populations in order to visualize similarities among the individual samples. Analogous to our analyses of qualitative differences, we performed a two-level nested ANOSIM (factors population and disease status nested within population) to test for quantitative differences in scent between healthy and diseased plants based on the Bray–Curtis similarity matrix. In addition, two-way SIMPER was used in Primer to determine the compounds being responsible for quantitative differences in scent that were found between healthy and diseased plants. Mann–Whitney *U* tests were calculated to test for differences in the total amount of the most variable scent compounds between healthy and diseased plants.

In all of the conducted ANOSIM analyses, 10,000 random permutations were run if possible in order to determine the level of significance.

The behavioral dual-choice tests were analyzed using the observed vs. expected frequency chi-square test in STATISTICA.

To test whether differences in floral scent among plants originating from different European and North American populations could explain part of the variation in fruit predation rates among these populations that had been observed in common garden studies, we used generalized linear models (STATISTICA 7.1) with a normal error distribution and an identity link function to test the effects of the continent of origin (class variable), population mean relative amount of the volatile compound (continuous variable), and their interaction on population mean predation rate (number of fruits predated divided by the number of fruits produced by all female plants).

Results

Qualitative Differences in Floral Scent Emission The flowers of both healthy and diseased plants emitted seven benzenoids, six monoterpenoids, three sesquiterpenoids, two phenylpropanoids, and a nitrogen-containing compound (Table 1).

Within populations, healthy and diseased plants emitted the same set of compounds, and there were no significant qualitative differences in scent (ANOSIM: disease status within population, $R=0.22$, $P=0.12$). Qualitative analyses showed that populations differed in the set of compounds that they emitted (ANOSIM: population effect, $R=0.58$, $P<0.01$), although most of the compounds, especially the monoterpenes [e.g., (*E*)-ß-ocimene and lilac derivatives], occurred in all populations and most of the samples. The benzenoid guaiacol was widespread, at least in healthy plants.

(Semi)quantitative Differences in Scent Emission The total amount of emitted scent strongly differed between healthy and diseased plants. The flowers of diseased plants emitted threefold lower amounts of scent than those of healthy plants (*U* test, $Z=2.9$, $P=0.003$).

Interestingly, flowers of diseased and healthy plants emitted not only different total amounts of scent but also different relative amounts of single compounds. The scent of healthy plants was dominated strongly by lilac aldehyde, reaching a median relative amount of 51% (Table 1). The median value of each of the other compounds was less than 10%. In diseased samples, lilac aldehyde also was the main component, contributing a median of 33% to the total scent emitted. However, in these samples, (*E*)-ß-ocimene also reached high relative amounts (median, 25%), and this compound was even the main constituent in some of the samples (maximum, 76%).

Quantitative analyses based on the total amount of individual compounds emitted by single flowers showed significant differences in scent between healthy and diseased plants (ANOSIM: disease status within population: $R=0.55$, $P=0.03$), whereas in this quantitative analysis, differences among populations were not significant (ANOSIM: Population effect, $R=0.20$, $P=0.12$). The disease status effect is visualized in a nonmetric multidimensional scaling plot (Fig. 2), where, in six out of the seven populations, healthy plants have lower mean scores for the dimension plotted along the *X*-axis than diseased plants from the same population. A SIMPER analysis revealed which compounds were responsible for the observed differences: five compounds each contributed at least 5% to the differences, and together they explained 78% of the differences between healthy and diseased plants across the different populations. Lilac aldehyde was most variable and explained 50% of the differences, whereas the contribution of the other four compounds was relatively small (6–9%). The total amount of lilac aldehyde was significantly reduced in diseased plants, and this was also true for arbusculone and a lilac aldehyde degradation

Table 1 Total amount of scent trapped, occurrence and mean relative amount of floral scent compounds found in samples of 12 healthy and 14 diseased flowers (both healthy and diseased samples were collected from seven populations each)

	Healthy				Diseased			
	Occurrence s/p	Median	Quartile	Minimum–maximum	Occurrence s/p	Median	Quartile	Minimum–maximum
Total amount of scent trapped (ng 3 min^{-1})		41.1	22.3–68.4	14.4–94.3		13.2	5.8–29.5	2.2–36.8
N-bearing compounds								
3-Methylbutylaldoxime[a]	6/4	0.1	0–1.4	0–11.3	7/3	0.7	0–14.8	0–43.8
Monoterpenoids								
α-Pinene[a]	5/4	0	0–0.4	0–1.0	5/3	0	0–0.5	0–3.7
(*E*)-ß-Ocimene [a]	11/7	9.9	4.5–15	0–18.8	14/7	24.6	5.9–42.4	3.2–75.6
(*Z*/*E*)-Arbusculone	11/7	7.2	4.6–10.4	0–11.7	8/6	0.1	0–0.9	0–18.4
Lilac aldehyde[a]	12/7	51.0	37.2–61.1	17–72.5	12/7	33.4	7.4–46.6	0–56.7
Lilac alcohol[a]	11/7	1.1	0.7–1.8	0–5.0	12/7	4.2	0.5–10.2	0–34.5
Lilac degradation	10/7	4.5	2.1–6.3	0–8.1	5/4	0	0–2.5	0–4.2
Sesquiterpenoids								
α-Longipinene	3/3	0	0–tr	0–0.9	2/1	0	0–0	0–3.1
Longicyclene	3/3	0	0–tr	0–0.9	3/2	0	0–0	0–1.1
Longifolene	4/3	0	0–0.1	0–1.0	3/2	0	0–0	0–1.5
Benzenoids								
Benzaldehyde[a]	9/6	0.4	tr–5.6	0–15.8	8/5	0.6	0–2.2	0–19.5
Phenylacetaldehyde[a]	5/4	0	0–18.8	0–54.9	4/3	0	0–7.3	0–27.2
Guaiacol[a]	11/7	1.7	0.6–2.8	0–7.8	6/5	0	0–0.2	0–5.3
Methylbenzoate[a]	5/3	0	0–1.7	0–38.4	4/3	0	0–tr	0–44.5
2-Phenylethanol[a]	2/2	0	0–0	0–2.9	3/2	0	0–0	0–4.9
Methyl salicylate[a]	8/5	1.0	0–8.8	0–24.5	8/4	1.3	0–6.2	0–24.3
Benzyl benzoate[a]	1/1	0	0–0	0–7.6	4/3	0	0–tr	0–3.2
Phenylpropanoids								
(*E*)-Cinnamaldehyde[a]	1/1	0	0–0	0–1.2	3/2	0	0–0	0–5.1
(*E*)-Cinnamyl alcohol	2/2	0	0–0	0–2.5	2/2	0	0–0	0–10.8

s number of samples, *p* number of populations, *tr* the amount was less than 0.05%

[a] Identification is based on authentic standard compounds

product (Fig. 3). The amount of phenylacetaldehyde and (*E*)-ß-ocimene did not differ between healthy and diseased plants.

Electrophysiological Responses When testing antennal responses of *H. bicruris* to floral scent collected from healthy and diseased plants from different populations, clear signals were elicited in each run. In total, antennae responded to 19 different compounds. Clear responses in the antennae were elicited by (*E*)-ß-ocimene and/or phenylacetaldehyde, 2-phenylethanol, different lilac aldehyde stereoisomers, methyl salicylate, (*E*)-cinnamyl alcohol, and benzyl benzoate (Fig. 4). Some compounds, such as phenylethyl acetate and (*E*)-cinnamyl alcohol, did not occur in all samples and consequently resulted in differences in the antennal responses among populations. Within populations, no consistent differences in antennal responses between healthy and diseased plants were observed, indicating that antennae responded to the same set of compounds in samples collected from healthy and diseased plants.

Behavioral Tests In the first behavioral experiment, we offered two flowers with volatile profiles that were qualitatively similar, and these flowers also emitted compounds in similar relative amounts (both mainly containing isoprenoids, especially lilac aldehyde) but that were quantitatively different (Fig. 5a). The moths were more attracted to flower 2, which emitted a higher amount of volatiles than flower 1 (nine females and seven males vs. one female and one male). In the second experiment, moths were offered a choice between two flowers that, in addition to quantitative differences, represented quite different chemotypes (Fig. 5b). Flower 3 was dominated by

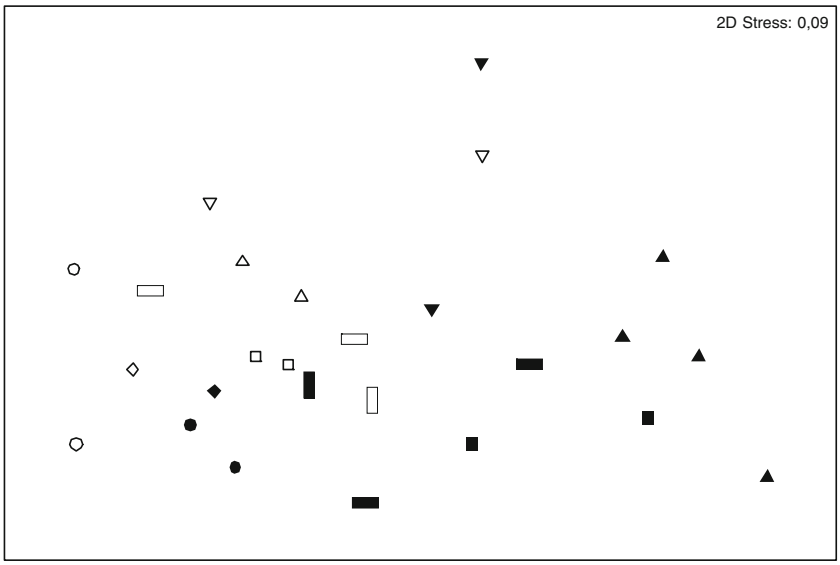

Fig. 2 Multidimensional scaling of the floral scent profile of 12 healthy (*open symbols*) and 14 diseased (*corresponding solid symbols*) flowers from seven different populations of *Silene latifolia* based on Bray–Curtis similarities, which were built on the basis of the total amount of the scent compounds emitted. The origins of the plants used for scent collection is given in Fig. 1

benzenoids, mainly phenylacetaldehyde (only small amounts of lilac aldehyde were emitted); flower 4 was dominated by lilac aldehyde. The moths were attracted equally to both flowers, even though flower 3 emitted about twice the amount of volatiles compared to flower 4 (four females and five males vs. three females and five males).

Associations Between Volatile Compounds and Fruit Predation Of the 21 compounds to which *H. bicruris* showed antennal responses (present study and Dötterl et al. 2006), only lilac aldehyde, which was also the quantitatively most important compounds, could explain a significant part of the variation in fruit predation by this moth among plant populations (Fig. 6a). Plants from North American populations suffered higher fruit predation than plants from European populations [$X^2(1)=11.1$, $P<0.001$] independently of the mean population level of lilac aldehyde. However, within both European and North American populations, predation rate increased with the mean population level of lilac aldehyde [$X^2(1)=3.83$, $P<0.05$, no interaction continent×lilac aldehyde $X^2(1)=0.37$, $P=0.54$]. The amounts of 2-phenylethanol and phenylalcetaldehyde, which are negatively correlated with the amount of lilac aldehyde at the population level, tended to show a negative association with predation rate [$X^2(1)=2.93$ and 2.57, respectively, $P<0.10$; Fig. 6b, c].

Discussion

Healthy vs. Diseased Plants Within the *Silene–Hadena* nursery pollinator system, *H. bicruris* has a strong oviposition preference for healthy over diseased (i.e., anther-smut infected) flowers of *S. latifolia* (Biere and Honders 2006), which is likely an adaptive response (Biere et al. 2002). The data in this study show that there are no qualitative differences (i.e., identity of compounds) in scent emitted by healthy and diseased plants, indicating that both groups of plants emit the same compounds. In the electroantennographic assays, *Hadena* responded to the same compounds in healthy and diseased plants. However, flowers of diseased plants emitted only about one third of the amount emitted by healthy flowers, and there were also differences in the relative (percentage) amount of compounds emitted. Most prominently, the diseased plants had a decreased relative amount of lilac aldehyde and, because the total amount of (*E*)-ß-ocimene was not affected by the disease status of plants, a higher relative amount of (*E*)-ß-ocimene. These differences in scent between healthy and diseased plants may be used by *H. bicruris* to discriminate between them.

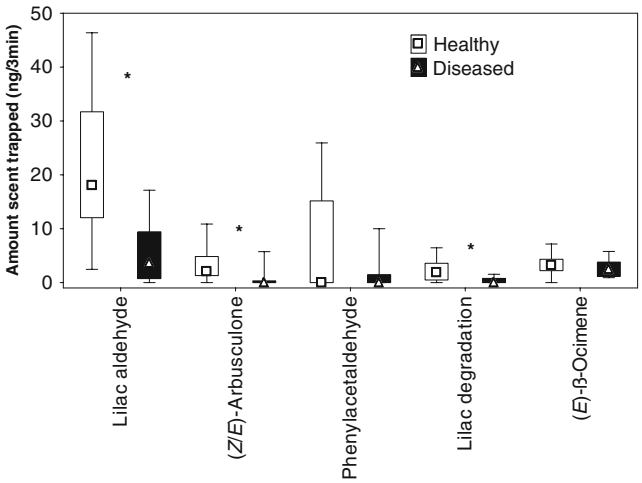

Fig. 3 Amount of *Silene latifolia* floral scent (median, 25%–75%, minimum to maximum) trapped for the compounds being most variable (according to a two-way SIMPER analysis) among healthy and diseased plant individuals across all population groups (*$P<0.01$, *U* test)

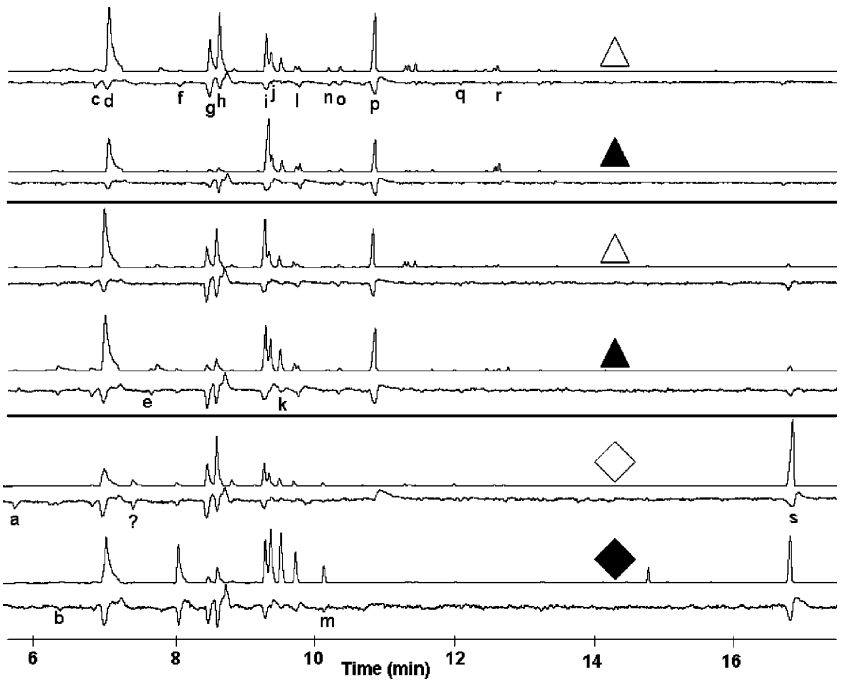

Fig. 4 Antennal responses of *Hadena bicruris* females (*lower lines*) to six different floral scent samples (*upper lines*) of healthy (*open symbols*) and diseased (*solid symbols*) plants of two different populations (see also Fig. 1 for the origin of the plants). *a* Benzaldehyde, *b* (*Z*)-3-hexenyl acetate, *c* benzyl alcohol, *d* (*E*)-ß-ocimene/phenylacetaldehyde, *e* guaiacol, *f* 2-phenylethanol, *g* lilac aldehyde A, *h* lilac aldehyde B+C, *i* methyl salicylate, *j* lilac alcohol A, *k* lilac alcohol B+C, *l* benzenepropanol, *m* phenylethyl acetate, *n* (*Z*)-cinnamyl alcohol, *o* (*E*)-cinnamyl aldehyde, *p* (*E*)-cinnamyl alcohol, *q* benzyl isovalerate, *r* (*E*)-cinnamyl acetate, *s* benzyl benzoate

One possible reason for the different effects of the disease on the total and relative amounts of lilac aldehyde and (*E*)-ß-ocimene is that the biosynthesis of monoterpenes is differentially affected by anther smut infection. Both monoterpenes are synthesized in the plastids via geranyl pyrophosphate (GPP) in the methylerythtritol phosphate pathway (Dudareva and Pichersky 2000). (*E*)-ß-Ocimene is built directly from GPP by an ocimene synthase (Bohlmann et al. 2000), while lilac aldehyde is synthesized via linalool, which is oxidized to 8-hydroxylinalool and 8-oxolinalool (these compounds were not found in the scent samples), and finally cyclized to lilac aldehyde (Kreck et al. 2003). Until now, only linalool synthase, the enzyme converting GPP to linalool has been identified (Pichersky et al. 1994), whereas the other enzymes involved in synthesis of lilac aldehyde via linalool are unknown. Because of differences in synthetic pathways, the anther smut fungus could have affected the steps leading from GPP to lilac aldehyde but not the step from GPP to (*E*)-ß-ocimene.

An alternative explanation for the effect of anther smut infection on floral scent is that changes in the total and relative amounts of lilac aldehyde and (*E*)-ß-ocimene are indirect effects of morphological changes that follow fungal infection due to direct interference of infection with steps in the biosynthetic pathway. Within *S. latifolia* flowers, lilac aldehyde is emitted from the anthophore, a column-like structure at the base of the ovary, whereas (*E*)-ß-ocimene is emitted from the petals (Dötterl and Jürgens 2005). In diseased plants, the ovary size is dramatically reduced, whereas petal size is only moderately reduced (Shykoff and Kaltz 1998; Biere and Honders 2006). Therefore, it would

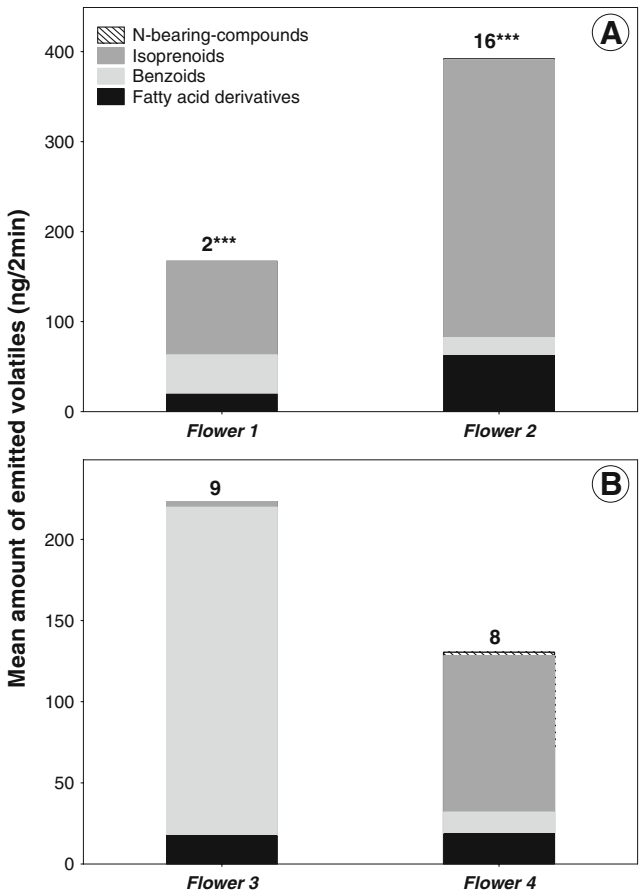

Fig. 5 Attraction of *Hadena bicruris* to different scenting flowers of *Silene latifolia* (*Flowers 1* to *4*). The *numbers above the columns* indicate the number of *H. bicuris* individuals attracted to a particular flower. In total, 21 specimens were tested in choice-test **A** and choice-test **B**, respectively (***$P<0.001$, chi-square test)

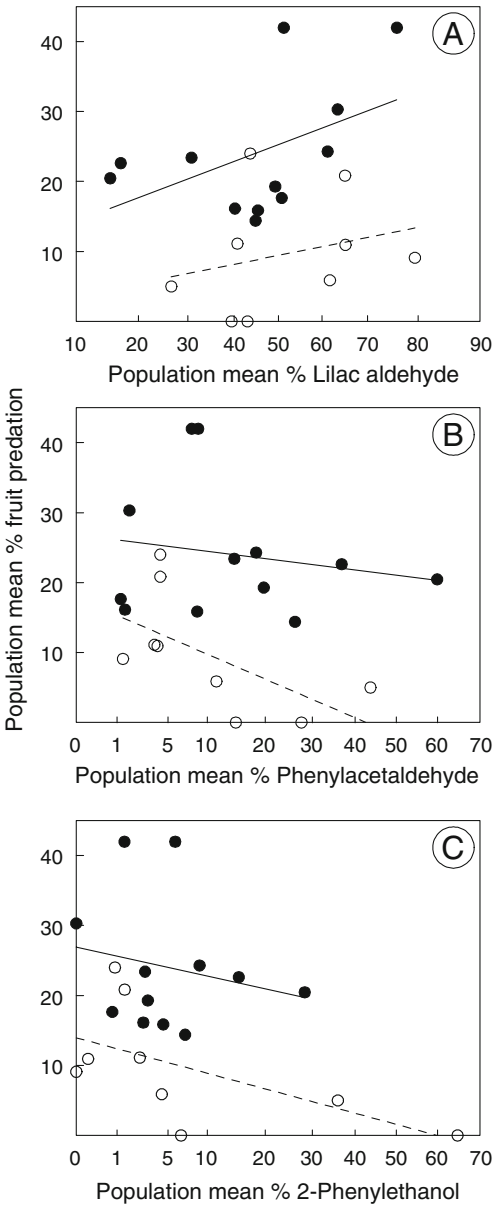

Fig. 6 Population mean fruit predation rate (%) as a function of population mean relative amount of scent volatiles: **A** lilac aldehyde, **B** phenylacetaldehyde, **C** 2-phenylethanol. Populations originating from North America (*closed dots*, *solid regression lines*) and from Europe (*open dots*, *dashed regression lines*) are indicated by different symbols. Note the arcsine-square root scale of the *X*-axes

be interesting to investigate whether the size of the anthophore is correlated with that of the ovary and whether the reduced amount of lilac aldehyde emitted from diseased flowers resulted from stunted development of both the ovary and the anthophore. The relatively small impact of fungal infection on petal size does not affect the levels of (*E*)-ß-ocimene emission.

Lilac aldehyde, as a single compound, features prominently in the *Hadena–Silene* interaction in that it is as attractive to *Hadena* moths as is the total blend of volatiles emitted by *S. latifolia* flowers (Dötterl et al. 2006). This suggests that the reduced predation rate of *H. bicruris* in diseased plants (Biere and Honders 2006) could be due mainly to the reduced total amount of emitted lilac aldehyde. Indeed, the behavioral tests conducted in this study and in previous studies demonstrate that scent quantity (both absolute amount of single compounds, such as lilac aldehyde, and total amount of floral scent) is an important factor in the attraction of *H. bicruris* (Fig. 5, see also Dötterl et al. 2006). Given that lilac aldehyde also is an effective attractant for other visitors/pollinators of *S. latifolia*, such as the generalist noctuid moth *Autographa gamma* (Plepys et al. 2002), diseased plants may be less attractive to flower visitors in general. Indeed, nocturnal flower visitors tend to prefer healthy over diseased plants (Shykoff and Bucheli 1995).

The reduced attractiveness of fungus-infected flowers to flower visitors would seemingly represent an adaptive dichotomy when viewed from both the fungus and the plant's perspective. It would be disadvantageous from the perspective of the fungus *M. violaceum*, which needs pollen vectors to ensure spore transmission, but may be advantageous for *S. latifolia* plants, as it reduces the rate of infection in their offspring (plants usually produce healthy pollen or seeds before becoming infected) or in their healthy kin. However, the lower attractiveness of fungus-infected flowers might have a positive effect on fungus spore dispersal within plant populations if moths that visit infected plants also visit fewer flowers on these plants (since they are less attracted), thereby reducing the waste of spores deposited on other flowers of already infected plants and increasing the number of visits to uninfected individuals. Additional research is needed to investigate the effect of reduced odor emission on the flower visitation patterns of moths. Another possible benefit to the fungus is the change in floral scent, as suggested by the fact that several volatile compounds are fungicidal, including lilac aldehyde (Komaki et al. 2005). Therefore, a decreased emission of lilac aldehyde may, in spite of the decreased visitor attraction, have a positive effect on the fitness of the fungus.

Plant pathogenic fungi, other than *M. violaceum*, that depend on insects for the transmission of their spores/gametes have been reported to affect the scent of their host plants, but mainly through the emission of additional volatiles rather than through a decrease in emission rates. In fungi that infect vegetative plant parts, volatiles emitted by the fungus itself or by infected host plants appear to be involved in attraction of vectors in *Berberis vulgaris* infected by *Puccinia arrhenatheri* (Naef et al. 2002), in *Arabis drummondii* infected by *Puccinia monoica* (Roy and Raguso 1997; Raguso and Roy 1998), and in *Brachypodium* and *Anthoxanthum* grasses infected by *Epichloë*

species (Schiestl et al. 2006). Interestingly, in our analyses of *Silene* flowers, we found no additional fungal or fungal-induced compounds, but this may be due to the location of spore release, i.e., in flowers as compared to vegetative parts. In the *Silene–Microbotryum* interaction, spores are released in the flower, which is already attractive for the spore-transmitting insects that visit the flowers, and no additional fungus-derived scent or modification of flower scent may be required to attract vectors. In the other systems, discussed above, spores are released from vegetative regions (e.g., leaves), which usually have no inherent attractiveness to insects that transmit the spores, and consequently, new volatile compounds are needed to ensure visitation of these plant parts.

Volatile Compounds and Fruit Predation in Healthy Plants The plant *S. latifolia* shows high intraspecific variation in flower scent profiles (Dötterl et al. 2005b, 2007), part of which can be attributed to variation between populations (Dötterl et al. 2007). Most plants and populations are dominated by lilac aldehyde, but in some, the relative amount of lilac aldehyde is low, and the scent is dominated instead by other compounds, such as phenylacetaldehyde (Dötterl et al. 2005b, 2007). Little is known about factors that maintain floral scent variability in *S. latifolia* or the role of different volatile blends in local interactions of *S. latifolia* with its pollinators and fruit predators. However, the data demonstrate that populations that emit lower relative amounts of lilac aldehyde also have lower predation rates by *Hadena* and, therefore, have supposedly lower flower visitation rates, which is supported by our dual-choice behavioral tests demonstrating the importance of lilac aldehyde for the attraction of *Hadena*. Therefore, we hypothesize that differences in predation rates found both among European and North American populations could just be a function of the average amount of lilac aldehyde emitted by the plants in these populations.

Interestingly, floral scent profiles of individual flowers could not explain the generally higher fruit predation rates of plants originating from North America compared to plants from Europe (Wolfe et al. 2004). For any given relative amount of lilac aldehyde, plants from North American populations suffered higher predation rates than those from European populations (Fig. 6a). This observation does not necessarily imply that flower volatiles play no role in intercontinental differences in predation rate. Although absolute and relative amounts of volatile compounds did not show large differences between flowers from North American and European plants at the level of individual flowers (Dötterl et al. 2005b), the number of flowers produced by plants originating from North American populations is about three times higher than that of their European counterparts (Blair and Wolfe 2004; Wolfe et al. 2004). At the whole plant level, North American plants emit larger amounts of flower volatiles than European plants. As scent quantity is important for attraction of *H. bicruris* (Fig. 5; Dötterl et al. 2006), North American plants generally may be more attractive for *Hadena* than plants originating from Europe, resulting in higher predation rates of flowers.

It is tempting to speculate that populations with low relative amounts of lilac aldehydes might occur in areas in which larger suites of alternative pollinators are available, making plants less dependent on *Hadena* for pollination and driving selection for lower amounts of lilac aldehydes to avoid *Hadena* fruit predation. Studies in other nursery pollinator systems indeed have shown that the outcome of co-evolutionary interactions can show extreme geographic variation (Thompson and Cunningham 2002). However, the limited information that is currently available on the interactions between *Silene* and alternative pollinators suggests that other, more generalistic, insect pollinators might use lilac aldehydes as attractants as well, which implies that *S. latifolia* plants with low amounts of lilac aldehyde may not only enjoy lower predation rates but may also suffer lower pollination success. Therefore, more studies that investigate the reproductive success of plants emitting different patterns of flower volatiles are needed to get insight in the adaptive significance of floral scent variability in *S. latifolia*.

The marginally significant negative association we found between fruit predation rate and the relative amounts of phenylacetaldehyde and 2-phenylethanol, two compounds that commonly occur in many floral scent samples of *S. latifolia* (Dötterl et al. 2005b, 2007), may be just an indirect effect mediated by the negative correlation between the relative amounts of these two compounds and the relative amount of lilac aldehyde.

Attraction and Oviposition Although differences in predation/oviposition rate by *H. bicruris* between healthy and diseased plants might, similarly to attraction rates, be explained by differences in floral scent, we do not know whether we should consider floral scent as a cue that elicits the oviposition behavior in *H. bicruris* or not. In other Lepidoptera, oviposition stimuli have been identified to be non-volatile compounds (e.g., Feeny et al. 1988; Haribal and Renwick 1996; Honda et al. 2001). Thus, it seems that generally volatile compounds are used for finding host plants, whereas less- or non-volatile compounds are used for host-plant recognition and for eliciting oviposition behavior (Honda 1995). Chemotactile receptors of the tarsi and the ovipositor are often involved in perception of such oviposition cues.

In *Hadena*, chemotactile receptors may play a role in oviposition, and oviposition cues could be located on the

petal surface of *S. latifolia* flowers. Typically, after *Hadena* is attracted to a flower, it lands on the flower, drinks nectar, and then lays an egg (Brantjes 1976). Therefore, oviposition cues may be located not only on the petals but even inside the flower tube, where they could be perceived by receptors on the proboscis (see also Biere and Honders 2006). The attraction-active lilac aldehyde may play a role in oviposition as well, as this compound is emitted at the base of the gynoecium near the nectaries (Dötterl and Jürgens 2005). However, *H. bicruris* strongly prefers female over male flowers for oviposition, and lilac aldehyde is the dominant compound in the scent of both male and female flowers, with no difference in scent observed (e.g., Dötterl and Jürgens 2005). Thus, other cues must be involved in lieu of, or in addition to, lilac aldehyde.

In summary, the cues used by *H. bicruris* to discriminate between healthy vs. smut-infected plants of *S. latifolia* seem to point to lilac aldehyde in the moth's attraction to the flowers, but the cues used in oviposition need to be identified before we can understand fully the mechanism leading to different oviposition and predation rates in the *Silene–Hadena* interaction.

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Detoxification of Gramine by the Cereal Aphid *Sitobion avenae*

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Abstract Secondary metabolites play an important role in host plant resistance to insects, and insects, in turn, may develop mechanisms to counter plant resistance mechanisms. In this study, we investigated the toxicity of gramine to the cereal aphid *Sitobion avenae* and some enzymatic responses of *S. avenae* to this alkaloid. When *S. avenae* fed on an artificial diet containing gramine, mortality occurred in a dose-dependent manner. The LC_{50} of gramine was determined to be 1.248 mM. In response to gramine, *S. avenae* developed increased activities of carboxylesterase and glutathione *S*-transferase, two important detoxification enzymes. The activities of both enzymes were positively correlated with the concentration of dietary gramine. In addition, the activities of peroxidase and polypheolic oxidase, two important oxidoreductase enzymes in *S. avenae*, increased in response to gramine; however, catalase activity decreased when insects were exposed to higher levels of dietary gramine. The potential role of gramine in host plant resistance and *S. avenae* counter-resistance is discussed.

Keywords Gramine · *Sitobion avenae* · Carboxylesterase · Glutathione *S*-transferase · Peroxidase · Polyphenol oxidase · Catalase

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Introduction

Host plant resistance against insects is recognized as an important component of integrated insect pest management (Gao 1994; Schotzko and Bosque-pérez 2000; Li et al. 2001). Plant resistance is attributed largely to secondary metabolites such as phenolics, nonprotein amino acids, and alkaloids, which are documented to be deleterious to insect herbivores (Manuwoto and Scriber 1985; Ciepiela and Sempruch 1999; Wang et al. 2006).

Many alkaloids are strong deterrents to aphids and other herbivores and have been used as botanical insecticides and/or antixenotic chemicals (Luo et al. 1997; Jiang et al. 1999). Gramine, a simple indole alkaloid found in barley and other crop plants, occurs widely in plants of Gramineae and has a wide range of biological activities against insects, mammals, and bacteria (Corcuera 1984, 1993; Züñiga and Corcuera 1986; Leszczynski et al. 1989).

In the plant–insect ecosystem, insect detoxification enzymes (i.e., esterase and glutathione *S*-transferase) and oxidoreductases (i.e., polyphenol oxidase, peroxidase, and catalase) are important antiresistant agents against the secondary metabolites found in their host plants. Their role in overcoming plant resistance has been studied extensively in a number of insect species (Gao et al. 1997; Figueroa et al. 1999; Luo and Zhang 2003). However, little is known about the changes of detoxification enzymes and oxidoreductases in aphids in response to gramine.

The present study was conducted to elucidate some of the biochemical mechanism(s) of gramine in host plant resistance and to determine how the English grain aphid, *Sitobion avenae* (F.), counters this resistance. The specific objectives were (1) to assess the direct toxicity of gramine to *S. avenae* and (2) to determine activities of detoxification enzymes and oxidoreductases in *S. avenae* in response to gramine in its diet.

Methods and Materials

Insects *Sitobion avenae* nymphs and adults were collected from field-grown wheat and maintained on the aphid-susceptible cultivar "Beijing 411," under field conditions. Insects over two generations were used for experiments.

Diet Preparation and Gramine Toxicity To determine gramine toxicity to *S. avenae*, technical-grade gramine was incorporated into an artificial diet containing the following ingredients: 300 mg agrinine, 50 mg cysteine, 200 mg histidine, 200 mg lysine, 100 mg methionine, 100 mg phenylalanine, 100 mg serine, 100 mg tryptophan, 20 mg tyrosine, 10 mg ascorbic acid, 0.1 mg biotin, 50 mg choline chloride, 1.0 mg folic acid, 10 mg *p*-aminobenzoic acid, 10 mg nicotinic acid, 2.5 mg pyridoxine HCl, 2.5 mg thianine HCl, 500 mg K_3PO_4, 200 mg $MgCl_2.6H_2O$, 100 ml distilled water, and 25 g sucrose. The pH was adjusted to 6.0 with K_3PO_4 (Auclair 1965; Kieckhefer and Derr 1967; Chen et al. 2000). Gramine (Kingsley and Keith) was dissolved into the liquid artificial diet to get a range of concentrations (4.0, 2.0, 1.0, 0.5, 0.25, 0.125, and 0.0 mM) for LC_{50} determination.

To assess effect of gramine on *S. avenae* detoxification enzymes and oxidoreductases and to ensure sufficient live aphids for biochemical analyses, technical grade gramine was dissolved in the artificial diet to compose four different concentrations (0.025, 0.05, 0.1, and 0.2 mM) below the LC_{50}. A diet containing no gramine was used as the control.

The second–third instar nymphs of *S. avenae* were fed on the liquid diet held between two layers of Parafilm M® (Auclair 1965). Two layers of stretched Parafilm were placed in a transparent cylindrical Plexiglas tube (height 60 mm and diameter 22 mm) and covered on top with gauze to allow air and humidity exchange. After 72 h of feeding, mortality was determined, and the survivors were collected for analyses of the detoxification enzymes and oxidoreductases.

Detoxification Enzyme Assays Carboxylesterase (CarE) activity was measured according to the method of Ni and Quisenberry (2003), with modification. *Sitobion avenae* (50 mg) were homogenized in 0.02 M potassium phosphate buffer (2 ml; pH 7.0) at 0°C. The homogenate was centrifuged (5,000×*g* at 4°C for 15 min). An aliquot (0.1 ml) of the resulting supernatant was mixed with 0.9 ml of the solution containing a-NA and Fast blue RR salt (10 mg a-NA and 20 mg Fast blue RR salt dissolved in 2 ml acetone, and diluted to 25 ml by adding the above phosphate buffer). The reaction progress was monitored spectrophotometrically at 405 nm.

Glutathione *S*-transferase (GST) was measured according to Ni and Quisenberry (2003). Frozen *S. avenae* (50 mg) were homogenized in 0.1 M Tris–HCl buffer (2 ml; pH 8.0) at 0°C. The homogenate was centrifuged (10,000×*g* at 4°C for 15 min). The assay mixture contained the resulting supernatant (0.1 ml), the above described Tris–HCl buffer (1.4 ml), and reduced glutathione (40 mM; 50 μl). After a preincubation (5 min at 25°C), CDNB (30 mM; 60 μl) was added. Change in absorbance was monitored at 340 nm.

Oxidoreductase Assays Aphids (50 mg) from each sample were ground in liquid nitrogen and then homogenized in 1 M ice-cold potassium phosphate buffer (1 ml) containing 1% polyvinylpyrrolidone and 1% Triton X-100 at pH 7.0. The homogenate was centrifuged (10,000×*g* at 4°C for 15 min), and the supernatant was used for analyses of the following three oxidoreductases.

Peroxidase (POD) activity was measured using the method of Hildebrand et al. (1986) and Hori et al. (1997). Enzyme extract (20 μl) was mixed with the substrate containing hydrogen peroxide (30%; 10 μl), guaiacol (18 mM; 300 μl), and hydroxyethylpiperazine ethanesulfonic acid (HEPES) buffer in deionized water (200 mM; pH 7.0; 100 μl). POD activity was estimated from the increase in A_{470}. The measurement was repeated three times.

Polyphenol oxidase (PPO) activity was determined following the method of Hori et al. (1997). Enzyme extract (20 μl) was mixed with a solution containing 1.6% catechol in HEPES buffer (500 μl), 200 mM HEPES (pH 6.0; 100 μl), and deionized water (380 μl). PPO activity was estimated from the increase in A_{470}. The analysis was repeated three times.

Catalase (CAT) activity was determined as described by Hildebrand et al. (1986) with minor modification. Enzyme extract (20 μl) was mixed with a solution containing hydrogen peroxide (75 mM; 100 μl), HEPES (200 mM; pH 8.0; 100 μl), and deionized water (780 μl). CAT activity was estimated from the increase in A_{240}. The analysis was repeated three times.

Total protein content was determined by the Bradford method (Bradford 1976). Absorbance of the reaction mixture was read at 595 nm with a spectrophotometer, and protein content was determined from a standard curve established using known quantities of bovine serum albumin (from Sigma Chemical) and the protein assay reagent.

Statistical Analysis Toxicity of gramine to *S. avenae* was subjected to probit analysis using the PriProbit Program V1.6.3 (Sakuma 1998). Analysis of variance was used to analyze the activity data from GST, CarE, POD, PPO, and CAT assays in *S. avenae*. Means were separated by the least significant difference test (α=0.05) (SPSS 11.0).

Correlation between enzymatic activities and gramine concentrations was determined by correlation test (α= 0.05) (SPSS 11.0).

Results

Gramine Toxicity Gramine killed *S. avenae* when the insect was fed with the artificial diet containing higher concentrations of the alkaloid (Fig. 1). After 72 h of feeding, *S. avenae* mortality followed in a dosage-dependent manner. The LC_{50} was determined to be 1.248 mM (range 0.947–1.743 mM).

Effect of Gramine Enzyme Activity Total protein content of *S. avenae* varied after the aphids fed on the artificial diet containing gramine at various concentrations (Fig. 2). With the exception of aphids that fed on 0.025 mM gramine, higher levels of total protein were found in all treatments compared to the control (df=4, 10, F=275.34, P<0.001).

GST activity in *S. avenae* was influenced by artificial diets containing different amounts of gramine (df=4, 10, F=58.66, P<0.001) (Fig. 3 GST). GST activity was significantly greater in *S. avenae* that fed on the diet containing 0.05, 0.1, and 0.2 mM of gramine than on the control diet. A positive correlation was observed between gramine concentration in diet and GST activity in *S. avenae* (N=5; r=0.940; P=0.009).

CarE activity in *S. avenae* was also affected by the increased gramine in the diet (df=4, 10, F=150.16, P<0.001) (Fig. 3 CarE). Gramine concentration was positively correlated with CarE activity in *S. avenae* (N=5; r=0.889; P=0.022).

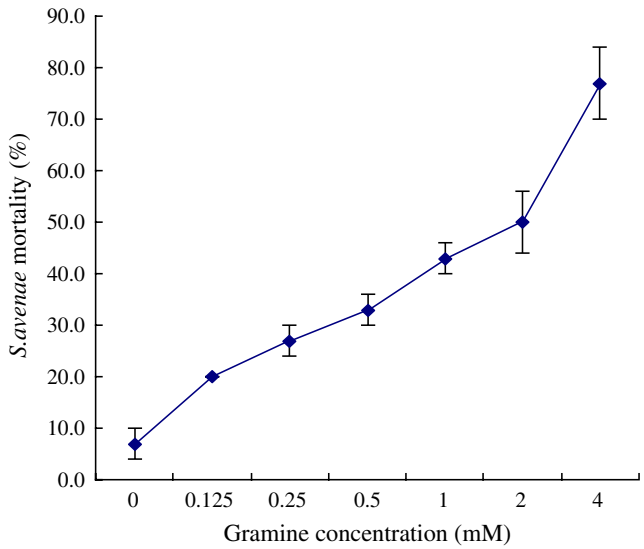

Fig. 1 Effect of gramine on the mortality of *S. avenae* at 72 h after treatment

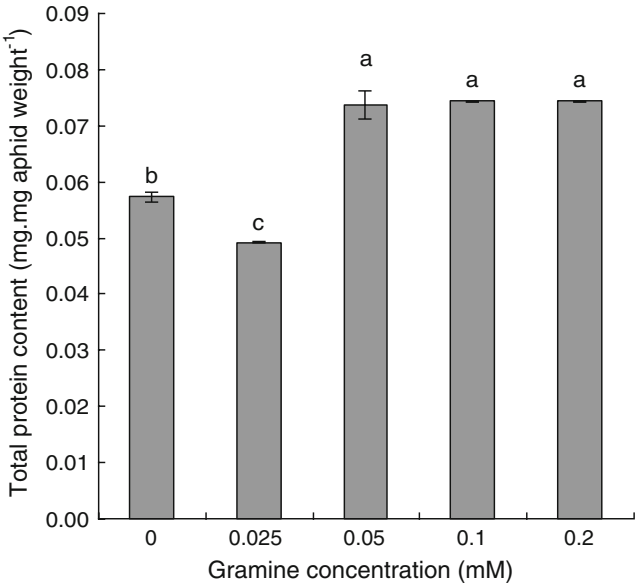

Fig. 2 Effect of different gramine concentrations on total protein content (mean ± SE) of *S. avenae*. *Bars with different letters* are significantly different (P<0.05)

POD and PPO activities were greater in *S. avenae* that fed on the artificial diet containing gramine than those on the control diet (df=4, 10; F_{POD}=113.20, F_{PPO}=57.74, P< 0.001) (Fig. 4 POD, PPO). Activities of both enzymes appeared to peak in *S. avenae* that fed on the diet containing 0.025 mM gramine. Then, enzymatic activities gradually increased with increased gramine (between 0.05 and 0.2 mM). PPO activity was positively correlated with dietary gramine levels (N=5; r=0.903; P=0.018). However, the correlation between POD activity and dietary gramine concentration was weak (N=5; r=0.800; P=0.052).

With the exception of 0.025 mM gramine treatment, CAT activity in *S. avenae* was suppressed by higher gramine concentration (between 0.05 and 0.2 mM) (df=4, 10; F=160.10; P<0.001) (Fig. 4 CAT). The correlation between dietary gramine concentration and CAT activity was not significant (N=5; r=−0.629; P=0.128).

Discussion

Many plant alkaloids are toxic to insects and are major resources of biological insecticides (Corcuera 1984; Jiang et al. 1999). Gramine has been considered as a deterrent and/or toxicant to many herbivorous insects such as *Schizaphis graminum*, *Rhopalosiphum padi*, *S. avenae*, and *Locusta migratoria* (Corcuera 1984; Zúñiga and Corcuera 1986; Leszczynski et al. 1989; Kanehisa et al. 1990; Rustamani et al. 1992; Moharramipour et al. 1997; Ishikawa and Kanke 2000; Wang et al. 2006). This study

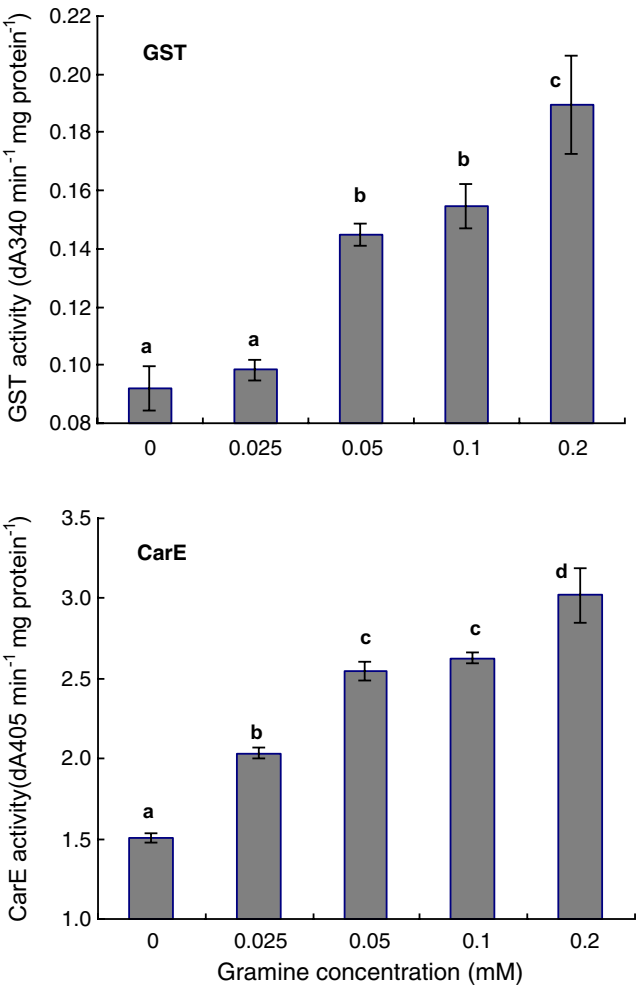

Fig. 3 Effect of gramine on activities (mean ± SE) of glutathione *S*-transferase (*GST*) and carboxylesterase (*CarE*) in *S. avenae*. Enzyme activities with *different letters* are significantly different ($P<0.05$)

showed that *S. avenae* mortality caused by exposure to dietary gramine was dosage-dependent with a defined LC_{50}, providing further evidence that gramine is active in plant resistance against aphids.

Detoxification enzymes (i.e., CarE and GST) play an important role in insect counter-resistance to plant secondary metabolites. The activity of these insects is closely related to the level of secondary metablites in host plants. CarE activity varies significantly among the populations of *Aphis gossypii* collected from different cotton varieties (Gao 1992; Jiang and Guo 1996). A higher CarE activity was observed in *R. padi* from resistant wheat varieties compared to susceptible varieties (Chen et al. 1997; Cai et al. 2004). Previous studies showed that alkaloids have been used as insecticides to regulate esterase activity in *Lipaphis erysimi* (Katenbach) and *Plutella xylostella* (L.) (Luo et al. 1997). The indole alkaloid gramine used in this study increased CarE activity in *S. avenae*, and the increase was positively correlated with dietary gramine concentrations, suggesting that CarE is involved with gramine detoxification.

GSTs (EC 2.5.1.18) constitute a group of enzymes that are involved in the detoxification of endogenous or

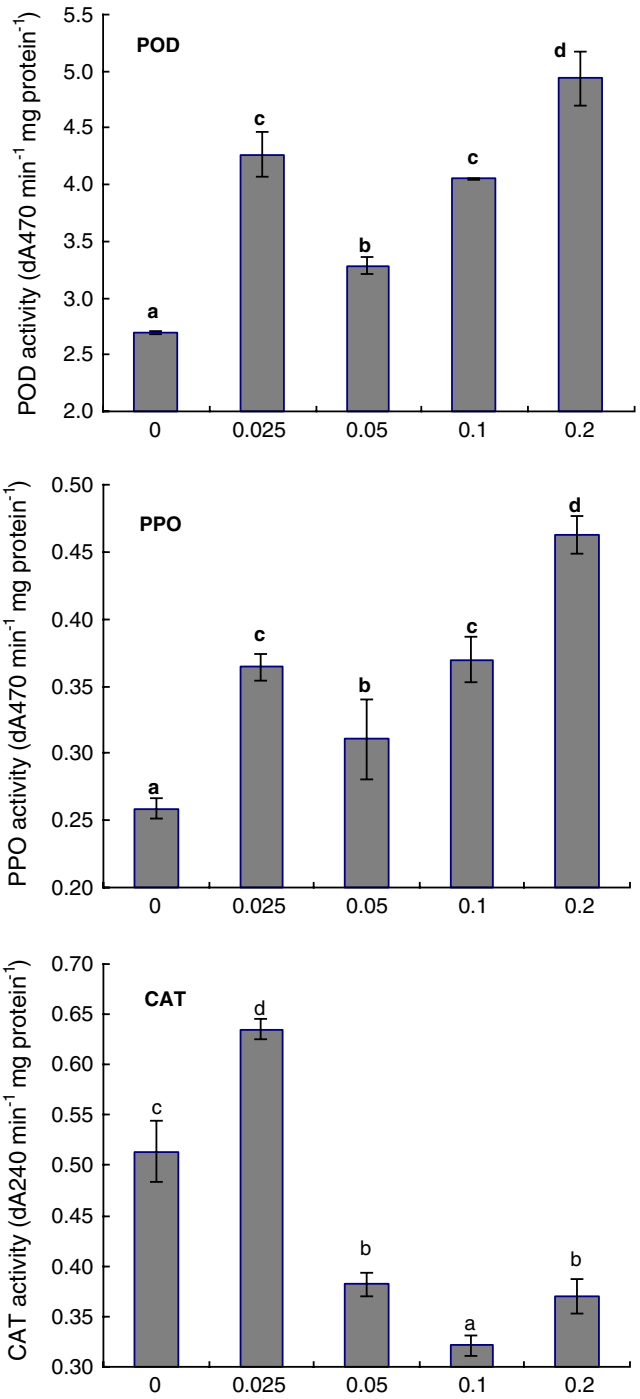

Fig. 4 Effect of gramine on activities (mean ± SE) of peroxidase (*POD*), polyphenol oxidase (*PPO*), and catalase (*CAT*) in *S. avenae*. Enzyme activities with *different letters* are significantly different ($P<0.05$)

exogenous toxic compounds (Vos and van Bladeren 1990). Increased level of GSTs in insects has been associated with resistance to a variety of insecticides (Foumier et al. 1992). Leszczynski et al. (1993) reported that GST activity was higher in *S. avenae* that fed on resistant wheat cultivars with high concentrations of phenolic compounds than those that fed on the susceptible ones. In this study, increased GST activity in aphids was observed when they fed on diets containing higher gramine concentrations, and enzyme activity was positively correlated with gramine concentration (Fig. 3). These results indicate that GSTs play a positive role in aphid antiresistance against allelochemicals (e.g., phenolics and gramine) in cereals.

POD and PPO are oxidoreductases, and in phytophagous insects, they may be able to counter plant resistance by reducing plant phenolics and their derivatives (Urbanska and Leszczynski 1992; Leszczynski et al. 1993). Phenolic compounds are involved in the resistance of cereal against aphids (Leszczynski et al. 1985, 1996). When aphids fed on a diet containing different concentrations of gramine, significantly different activities of POD and PPO were detected among the concentrations, and the gramine concentrations were correlated strongly with PPO activity in aphids but weakly with POD activity (Fig. 4). These results suggest that both POD and PPO in aphids are involved in gramine metabolism.

CAT is the classical enzyme in aerobic organisms that catalyzes the decomposition of hydrogen peroxide. This enzyme occurs in the midgut of *S. avenae* (Felton and Duffey 1991; Urbańska 2007). Many plant phenolics enhance the enzymatic activity in *S. avenae* (Figueroa et al. 1999; Loayza-Muro et al. 2000; Lukasik 2007). Interestingly, the opposite was observed in the present study, as *S. avenae* CAT activity was strongly reduced by higher gramine concentrations. However, significant correlations were not found between the activity and gramine concentration. This suggests that *S. avenae* CAT may be dissimilarly modified by the different cereal allelochemicals, i.e., alkaloids vs. phenolics.

In summary, gramine is considered to be an important secondary metablite in host plant resistance. This study showed that dietary gramine was toxic to *S. avenae*. In response to gramine, *S. avenae* enhanced CarE and GST activities, as well as PPO and POD activities, thus possibly countering the resistance.

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Do Caterpillars Secrete "Oral Secretions"?

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Abstract The oral secretions or regurgitant of caterpillars contain potent elicitors of plant induced responses. These elicitors are recognized by host plants to differentiate between simple mechanical injury and the presence of herbivores. In some cases, this level of recognition is highly specific. Despite the in-depth chemical characterization of these elicitors, little is known about the amounts delivered in regurgitant during feeding. In this study, we use a fluorescent dye to label regurgitant in order to visualize caterpillar regurgitation during feeding. The procedure is highly sensitive and allows us to visualize nanoliter amounts of regurgitant. We examined the propensity of larval *Helicoverpa zea*, *Heliothis virescens*, *Spodoptera exigua*, *Spodoptera frugiperda*, and *Manduca sexta* to regurgitate on various host plants. These species were selected because they have been among the most intensely studied in terms of elicitors. Our results indicate that most larvae did not regurgitate following a brief feeding bout (~10 min) during which they ate ca. 0.40 cm^2 of leaf. When larvae did regurgitate, it was typically less than 10 nl. This is several orders of magnitude less than is typically used in most studies on oral secretions. The frequency of regurgitation appears to vary depending upon the host plant. Larval *H. zea* are less likely to regurgitate when feeding on tomato leaves compared to corn mid-whorl tissue. Our results have importance in understanding the role of oral secretions in plant recognition of herbivory. Because caterpillars did not routinely regurgitate during feeding, it is likely that they avoid the elicitation of some plant defensive responses during most feeding bouts.

Keywords Elicitor · Regurgitant · Oral secretion · Plant signaling · Induced resistance · *Helicoverpa zea* · *Manduca sexta* · *Spodoptera exigua* · *Spodoptera frugiperda*

Introduction

The specificity of plant responses to herbivores is determined, in part, by the particular feeding style of the herbivore (Rodriguez-Saona et al. 2005) and by the presence of herbivore-derived elicitors or herbivore-associated-molecular-patterns (HAMPs) found in their secretions (Korth and Thompson 2006; Fatouros et al. 2008; Felton 2008; Felton and Tumlinson 2008; Mithöfer and Boland 2008). Lepidopteran secretions, which may contact the plant, include not only regurgitant from the insect gut but also saliva produced by the labial and mandibular salivary glands and feces (Felton 2008). Most of the HAMPSs that have been identified are found in the regurgitant or oral secretions (OS) of lepidopteran larvae and can elicit both direct and indirect defenses. These lepidopteran HAMPs include β-glucosidase (Mattiacci et al. 1995), fatty acid-conjugates (e.g., volicitin; Alborn et al. 1997; Voelckel and Baldwin 2004), and the inceptins (Schmelz et al. 2006) found in OS. HAMPs may trigger biosynthesis of wound hormones including jasmonic acid, the release of volatile emissions, and the induction of defense genes (Felton and Tumlinson 2008; Mithöfer and Boland 2008).

Another factor contributing to the specificity of plant responses to herbivores is that the activity of elicitors may be context specific. For example, the activity of elicitors such as volicitin or inceptins is host-plant specific (Schmelz

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et al. 2006, 2007). The transcriptome responses of two Solanaceous plants, *Nicotiana attenuata* and *Solanum nigrum*, to herbivory by *Manduca sexta* were strikingly different, although the fatty acid conjugates in OS of the caterpillar are not affected by diet (Schmidt et al. 2005). These data suggest that the elicitors are differentially active in the two host plants. Another plausible explanation for these differential responses is that although the composition of the elicitors in OS is not altered by host plant, there may be differences in the amount of OS applied to each host plant during feeding.

How the caterpillar applies these elicitors to the plant and in what amounts is much less understood. Volicitin found in the OS of caterpillars such as *Spodoptera exigua* has been well characterized and shown to induce volatile production in maize (Alborn et al. 1997). In an elegant study, radiolabeled volicitin was identified in the caterpillar regurgitant and recovered from corn leaves after feeding by *S. exigua* (Truitt and Pare 2004). The researchers estimated that 100 pmol of volicitin in 4.2 μl of regurgitant was transferred to the leaf over a 9-h period; although the authors noted that the secreted volicitin may arise from a mixture of fluids from the gut and salivary glands. Additionally, it is not clear if the volicitin originated solely from OS or also could have been deposited in fecal excrement.

Our laboratory has investigated the effect of host plant variation on the rate of salivary secretions in the noctuid *Helicoverpa zea*. We used antibodies targeted to specific components of caterpillar saliva to investigate this question. One of the active components in caterpillar saliva is the enzyme glucose oxidase (GOX; Eichenseer et al. 1999; Musser et al. 2002). Glucose oxidase, visualized on leaf tissue prints by using immunolabeling, is secreted at the feeding site and randomly across the leaf by larval *H. zea* (Peiffer and Felton 2005). GOX can be recovered from leaves fed on by larvae and then quantified by using the antibody. Larvae secrete microgram amounts of saliva depending upon the host plant (Peiffer and Felton 2005). Differences in rates of GOX secretion may partially explain the variation in responses of host plants to caterpillar salivation that we have observed (unpublished data).

Some researchers, however, have questioned the role oral secretions play in the specificity of plant responses and, instead, have argued that the physical damage inflicted by herbivores has not been adequately mimicked by investigators. In studies where oral secretions or saliva are tested, the mechanically wounded control treatment typically involves a single wounding event performed with a razor blade, pattern wheel, scissors, or other mechanical device (Alborn et al. 1997; Halitschke et al. 2001; Gomez et al. 2005; Schmelz et al. 2007; Wu et al. 2007). It has been consistently observed that a single wounding event may not adequately mimic the continuous or semicontinuous feeding by an herbivore (Mithöfer et al. 2005). A "mechanical" caterpillar (named MecWorm) was designed to test the effect of continuous feeding on the volatile emissions in lima bean leaves (Mithöfer et al. 2005; Arimura et al. 2008). Their experiments indicated that volatile emissions produced by MecWorm damage qualitatively resemble that produced by herbivore feeding (Mithöfer et al. 2005; Arimura et al. 2007), suggesting that OS are not required for volatiles released by caterpillar feeding.

Despite a fairly robust literature on caterpillar OS, no one has explicitly documented lepidopteran regurgitation on leaves during feeding. Regurgitant used in most studies is collected by squeezing the larva or even by applying an electrical shock. However, in certain caterpillar species, regurgitation is typically a defensive response to predators (Grant 2006; Brown et al. 2007). Thus, our objective was to determine the extent to which caterpillars may regurgitate during feeding. In this paper, we used a fluorescent dye to label the regurgitant, thereby allowing us to visualize and quantify regurgitant at the feeding site.

Material and Methods

Insects and Plants *H. zea*, *Heliothis virescens*, and *M. sexta* eggs were purchased from the North Carolina State University Insectary; *S. exigua* were kindly supplied by Jim Tumlinson, Penn State University; *S. frugiperda* were kindly supplied by Dawn Luthe, Penn State University. All caterpillars were reared on a wheat germ and casein-based artificial diet (Chippendale 1970) with ingredients purchased from BIOSERV (Frenchtown, NJ, USA). Insects were kept at 27°C, with a 16-h photoperiod.

Plants used were: tomato *Solanum lycopersicum* (cv Betterboy); corn *Zea mays* (cv B73); squash *Cucurbita pepo* (cv Dixie); and bean *Phaseolus vulgaris* (cv Bush Blue Lake 274). Before beginning the fluorescence experiments, we observed tomato leaves with the confocal microscope over a range of wavelengths. This preliminary scan indicated that leaves have minimal autofluorescence at 488 nm. Therefore, Alexa 488 was determined to be an appropriate dye to work with.

Effect of Alexa 488 on H. zea Larval Growth To determine the effect of Alexa 488 on caterpillar growth, 10 μg Alexa Fluor 488 carboxylic acid esters (Invitrogen, Carlsbad CA, USA) were dissolved in a small amount of water and then pipetted onto 1.0 g artificial diet. Fourth instar *H. zea* were allowed to feed for 3 day, then relative growth rates were calculated and compared to larvae feeding on diet without Alexa.

Sample Preparation and Observation of Feeding Sites by Confocal Microscopy To make regurgitant fluorescent, 10 μg Alexa Fluor 488 in 100 μl water were pipetted onto 0.5 g of artificial diet. Newly molted fifth instars were allowed to feed overnight on the spiked diet. Control caterpillars were fed diet to which 100 μl of water were added. By morning, the entire piece of diet had been consumed, and caterpillars were placed on leaves. After 10 min, caterpillars were removed, and the feeding site on the leaf excised and pressed between a glass slide and a coverslip and secured with tape. If caterpillars were allowed to feed longer, the ingested leaf material pushed the fluorescent diet through the gut, and the regurgitant became less fluorescent. Therefore, leaves were examined after only a single feeding bout in order to increase the chance of observing regurgitation.

To collect *H. zea* saliva, ice-chilled larva were immobilized in a metal hair clip and observed with a dissecting microscope. As the larva returned to room temperature, labial saliva was collected via capillary action into a gel loading pipet tip (VWR, West Chester, PA, USA) containing 3 μl water. Saliva and water were then expelled onto a glass coverslip and allowed to dry before observation. Ventral eversible gland (VEG) secretions were collected from the gland in noctuids in the same manner as saliva. Caterpillars were placed on parafilm and gently squeezed until they regurgitated. The regurgitant was collected, and 3 μl were placed on a coverslip and allowed to dry.

Leaves and coverslips containing secretions were observed with an Olympus FV1000 Laser Scanning Confocal Microscope (Olympus, America Inc., Melville, NY, USA) with an excitation of 488 nm. For each leaf, three visual fields were imaged at ×10 and relative intensity of fluorescence measured with FV10-ASW version 1.6. ANOVA using Fisher's separation of means was used to determine if the relative intensity of leaves were different from control leaves. Saliva and VEG secretions were imaged at a ×20 magnification.

Dilution Series and Standard Curve To determine the lowest amount of regurgitant detectable, regurgitant was collected from four larvae that had eaten Alexa 488 spiked diet. The regurgitant was diluted in water and 0.2 μl applied to detached leaves and allowed to dry. The leaves were mounted and observed as previously described. Relative intensity of fluorescence was plotted against amount of regurgitant applied to create a standard curve. This curve was used to estimate the amount of regurgitant caterpillars secreted onto the leaf.

Effect of Alexa Source Additional experiments were done to determine if *H. zea* would regurgitate differently if allowed to feed longer on plants, or if Alexa was acquired by feeding on spiked leaves rather than diet. To acclimate *H. zea* to tomato leaves, third instar *H. zea* were caged on intact 4-week-old tomato seedlings and allowed to feed. When larva molted to fifth instar, they were removed from plants and placed in cups with leaves spiked with Alexa 488. To spike the leaves, cut petioles were placed in 100 μl of solution containing 10 μg Alexa 488. When leaves had taken up the entire solution, they were fed to *H. zea*. After *H. zea* consumed the entire spiked leaf, they were placed on fresh leaves for 10 min, and leaf samples and secretions were prepared as previously described. The experiment was repeated with corn, except third instars were fed on detached corn leaves.

Effect of Spinneret Ablation on Regurgitation To determine if ablating the spinneret would effect regurgitation, before feeding on diet spiked with Alexa 488, *H. zea* caterpillars were ablated as previously described (Peiffer and Felton 2005). Ablation of the spinneret is a procedure routinely used to determine the role of labial gland saliva on induced defenses.

Results

Effect of Alexa 488 on H. zea Growth No differences were observed in relative growth rates of *H. zea*-fed Alexa-spiked diet vs. normal diet. *H. zea*-fed spiked diet had a relative growth rate of 0.5216 mg gained/day/mg larva, while those fed normal diet had a relative growth rate of 0.5284 mg gained/day/mg larva (F=0.34, P=0.562). This suggests that Alexa 488 has no obvious, deleterious effects on *H. zea* at the concentrations used.

Distribution of Fluorescence in Caterpillar Secretions After caterpillars had fed overnight on diet spiked with Alexa 488, we determined that their labial saliva and, in the case of the noctuid species, their VEG secretions were not fluorescent. No fluorescence was observed in the saliva of any *H. zea* tested (Fig. 1). The saliva of all eight *H. virescens* larvae observed lacked detectable fluorescence. Likewise, all eight *H. zea* and eight *H. virescens* lacked fluorescence in the VEG secretions. *M. sexta*, *S. frugiperda*, and *S. exigua* have comparatively short spinnerets, making it nearly impossible to collect saliva directly or to be assured that regurgitant is free of saliva. Thus, saliva was not specifically tested for these species. However, we were able to collect VEG secretions from ten *S. frugiperda* and nine *S. exigua*, and all lacked detectable fluorescence.

In all experiments, regurgitant, collected after the caterpillars ate spiked diet, was highly fluorescent (Table 1). Feces, from caterpillar-fed spiked diet, were also highly fluorescent (Fig. 1).

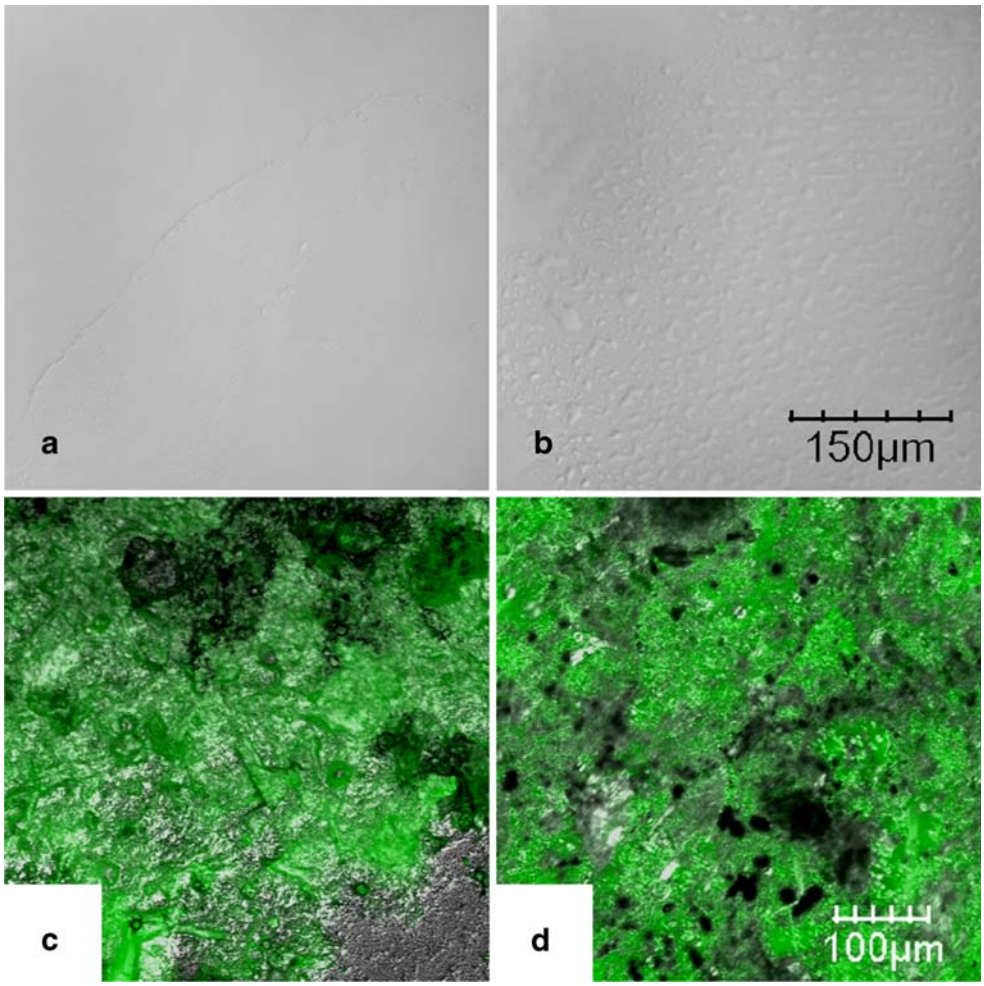

Fig. 1 Distribution of fluorescence in Helicoverpa zea oral secretions and feces after eating 0.5 g diet spiked with Alexa 488. **a** saliva, **b** VEG secretions, **c** regurgitant, **d** feces

Effect of Host Plant on Regurgitation in H. zea When *H. zea* larvae were placed on tomato leaves after feeding on spiked diet, they ate on average 0.12 cm^2 (SE=0.02) of leaf material before being removed. Fluorescence was detected on only one of 20 leaves (Fig. 2). Nineteen of the leaves lacked any fluorescence along the eaten edges or anywhere else on the leaf. The estimated average amount of regurgitant on this leaf was 0.32 nl (SE=0.12; Table 2).

H. zea ate on average 0.13 cm^2 (SE=0.07) of bean leaves, and regurgitant was visually detected on only one of five bean leaves (Table 2). *H. zea* ate 0.21 cm^2 (SE=0.11) of squash leaves, and regurgitant was detected on one of five leaves. When *H. zea* fed on corn leaves, they ate on average 0.26 cm^2 (SE=0.05) of leaf, and low levels of regurgitant were detected in five of eight leaves (Table 2). When *H. zea* were ablated before being fed spiked diet, they ate 0.31 cm^2 (SE=0.06), and regurgitant was detected on only one of six tomato leaves (Table 3).

Standard Curve for Regurgitant Applied to Leaf When serial dilutions of regurgitant were applied to leaves, the relative intensity of fluorescence and the amount of regurgitant applied were found to have a linear relationship (Fig. 2). From this standard curve, we estimated the amount of regurgitant on leaves with visually detected fluorescence.

Effect of Alexa Source on H. zea When *H. zea* were allowed to feed on leaves and then acquire Alexa from spiked leaves, they ate on average 0.22 cm^2/leaf (SE=0.09),

Table 1 Relative intensities of regurgitant from larvae fed diet spiked with Alexa 488 or fed control diet

Caterpillar species	Alexa 488 Diet	Control Diet
Helicoverpa zea	150.97	3.61
Heliothis virescens	192.51	13.62
Manduca sexta	55.29	14.27
Spodoptera exigua	22.72	1.19
Spodoptera frugiperda	86.05	0.10

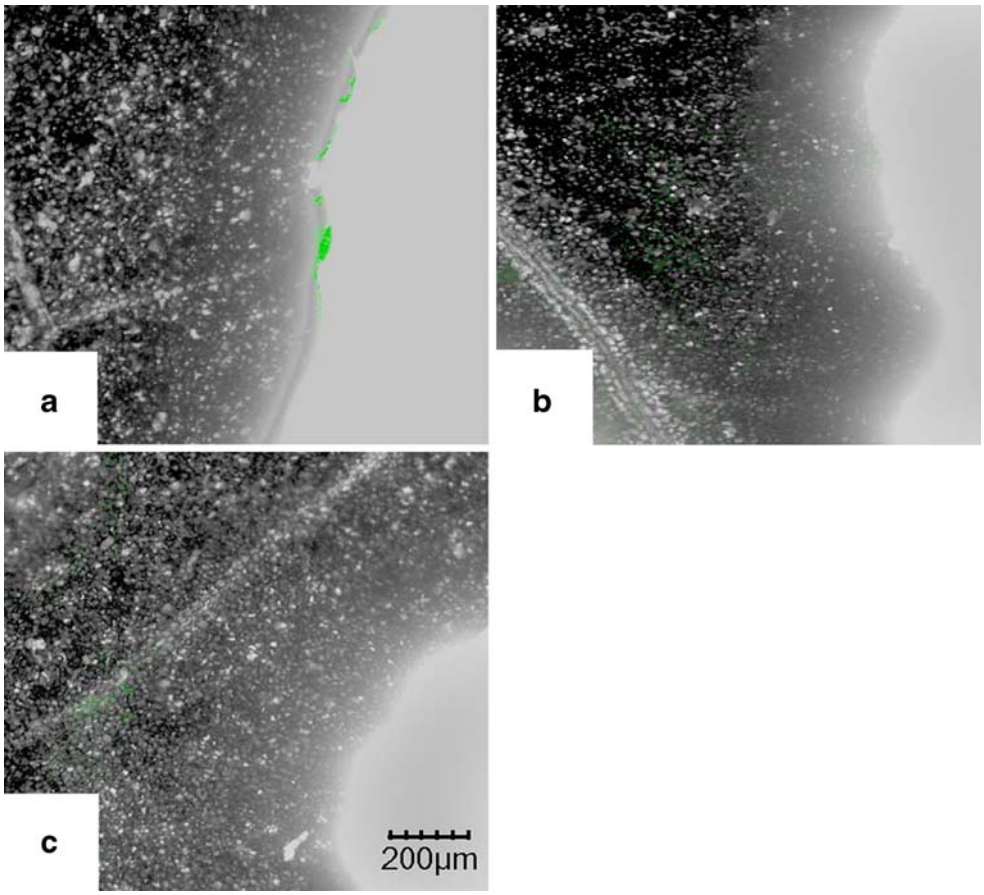

Fig. 2 Visual detection of fluorescent regurgitant from *Helicoverpa zea* after eating diet spiked with Alexa 488. **a** Tomato leaves with fluorescent regurgitant along the feeding site. **b** Leaf which *H. zea* fed upon but no regurgitant was detected. **c** Leaf fed upon by *H. zea* fed control diet

and fluorescence was not detected on any of the seven tomato leaves examined (Table 2). *H. zea* from corn ate 0.36 cm^2 (SE=0.10) of the corn leaf. Fluorescence was detected on only one of ten corn leaves after acquiring Alexa from corn leaves (Table 2).

H. virescens on Tomato Leaves H. virescens caterpillars had slightly higher rates of regurgitation, after eating 0.20 cm^2 (SE=0.02) leaf. When 14 tomato leaves were examined after being fed upon by caterpillars that had eaten spiked diet, fluorescence was detected on five (Table 3). A standard curve was generated from *H. virescens* regurgitant, and the average amount of regurgitant on the leaves was calculated to be 4.8 nl (SE=1.82).

Other Caterpillar Species When *M. sexta* larva were allowed to feed on diet spiked with Alexa 488, then tomato leaves, they ate on average 0.3 cm^2 (SE=0.10) of leaf material, and the collected regurgitant was highly fluorescent. However, fluorescence was not detected on any of 15 leaves following feeding (Table 3). This suggests that *M. sexta* do not typically regurgitate during feeding on tomato.

Table 2 Detection of *Helicoverpa zea* regurgitant on different host plants

Alexa source	Host plant	# Fluorescent/total # observed	Estimated amount of regurgitant (nl) mean ± SE
Diet	Tomato	1/20	0.32[a]
Diet	Bean	1/5	0.31[a]
Diet	Squash	1/5	0.27[a]
Diet	Corn	5/8	0.55±0.10
Tomato leaves	Tomato	0/7	0.00
Corn leaves	Corn	1/10	1.49[a]

[a] Result from one observation

Table 3 Detection of regurgitant on plants after feeding by caterpillars spiked with Alexa 488

Caterpillar species	Plant	# Fluorescent/total # observed	Estimated amount of regurgitant (nl) mean ± SE
Helicoverpa zea (ablated)	Tomato	1/6	0.07[a]
Heliothis virescens	Tomato	5/14	4.80±1.82
Manduca sexta	Tomato	0/15	0
Spodoptera exigua	Corn	3/9	0.90±0.34
Spodoptera frugiperda	Corn	2/10	1.20±0.49

[a] Result from one observation

Regurgitation on corn leaves was detected on three of nine leaves fed on by *S. exigua*, which ate 0.36 cm^2 (SE= 0.04) per leaf (Table 3). *S. exigua* regurgitant diluted and manually applied to corn leaves also gave a linear standard curve. From this curve, we determined that the three *S. exigua* that had visually detectable fluorescence had 1.6, 0.5, and 0.6 nl of regurgitant (Table 3).

S. frugiperda ate on average 0.32 cm^2 (SE=0.03) per leaf and two of ten *S. frugiperda* regurgitated on the corn leaves (Table 3). By using the standard curve generated from *S. exigua* data, the two *S. frugiperda* were calculated to each have secreted 1.4 and 1.0 nl of regurgitant.

Application of 20 μl Regurgitant Researchers studying the effects of regurgitant on plants typically apply 20 μl of regurgitant to wounded leaves. When we applied 20 μl fluorescent regurgitant to leaves, the fluorescence was obvious and bright. Relative fluorescence could not be measured because the detector was saturated at the settings normally used for the feeding experiments, and the area covered by fluorescence was too large to fit in a visual field (Fig. 3).

To determine if wounding of the plant tissue caused quenching or interference with the fluorescence detection, regurgitant was diluted 1:5 with water, and 20 μl were applied after wounding with a pattern wheel. Fluorescence was spread over the wounded surface and again very bright, coating not only the wound sites but also the entire leaf and trichomes (Fig. 4).

Discussion

Plants can discriminate herbivory from mechanical damage by either recognizing specific components (HAMPs) in the oral secretions of the herbivore and/or by the unique patterns of physical injury incurred during feeding (Howe and Jander 2008; Mithöfer and Boland 2008). Our laboratory has approached this problem by developing surgical techniques to prevent caterpillars from releasing saliva either by ablation of the spinneret (the secretory structure for labial gland saliva) or by surgically removing the labial glands (Musser et al. 2002, 2006). These techniques have demonstrated that saliva plays a key role in the suppression of inducible defenses in several caterpillar-host plant models (Musser et al. 2002, 2005; Bede et al. 2006; Delphia et al. 2006; Weech et al. 2008). The MecWorm was designed to more accurately mimic the continuous physical injury incurred during caterpillar feeding. MecWorm "feeding" produced volatile emissions qualitatively similar to actual caterpillar feeding on lima bean plants (Mithöfer et al. 2005). However, not all plant species respond to MecWorm treatment with a similar emission profile as the herbivore-induced suite (Maffei et al. 2007). This indicates that HAMPs may not be active in all plant species, and/or HAMPs may not be released by herbivores on all plant species. Nevertheless, there is strong evidence that certain HAMPs such as volicitin are important components in plant recognition of herbivores. Not only is volicitin secreted on maize leaves by *S. exigua* larvae (Truitt and Pare 2004) and activates specific indirect defenses (Alborn et al. 1997), but there is evidence for a putative volicitin receptor on the plasma membrane in maize (Truitt et al. 2004).

Because HAMPs have been identified in lepidopteron regurgitant, it has been assumed that as caterpillars feed, they regurgitate onto the wound site, thereby exposing the plant to the HAMPs. However, in order to better understand how plants recognize herbivores, it is necessary to determine whether regurgitant or OS are typically secreted during feeding bouts and if there is variation in the secretion depending upon a particular host plant species used by the herbivore. In the majority of studies on OS in caterpillars, researchers have routinely applied 10–20 μl of OS to leaves mechanically wounded without regard to the actual amounts that may be secreted. Our data indicate that regurgitation during feeding does not occur with every feeding bout, and when it does occur, caterpillars regurgitate in nanoliter amounts.

We used Alexa 488, which is a highly fluorescent and very stable dye, to visualize and quantify regurgitation. Caterpillars placed on diet spiked with Alexa 488 have highly fluorescent

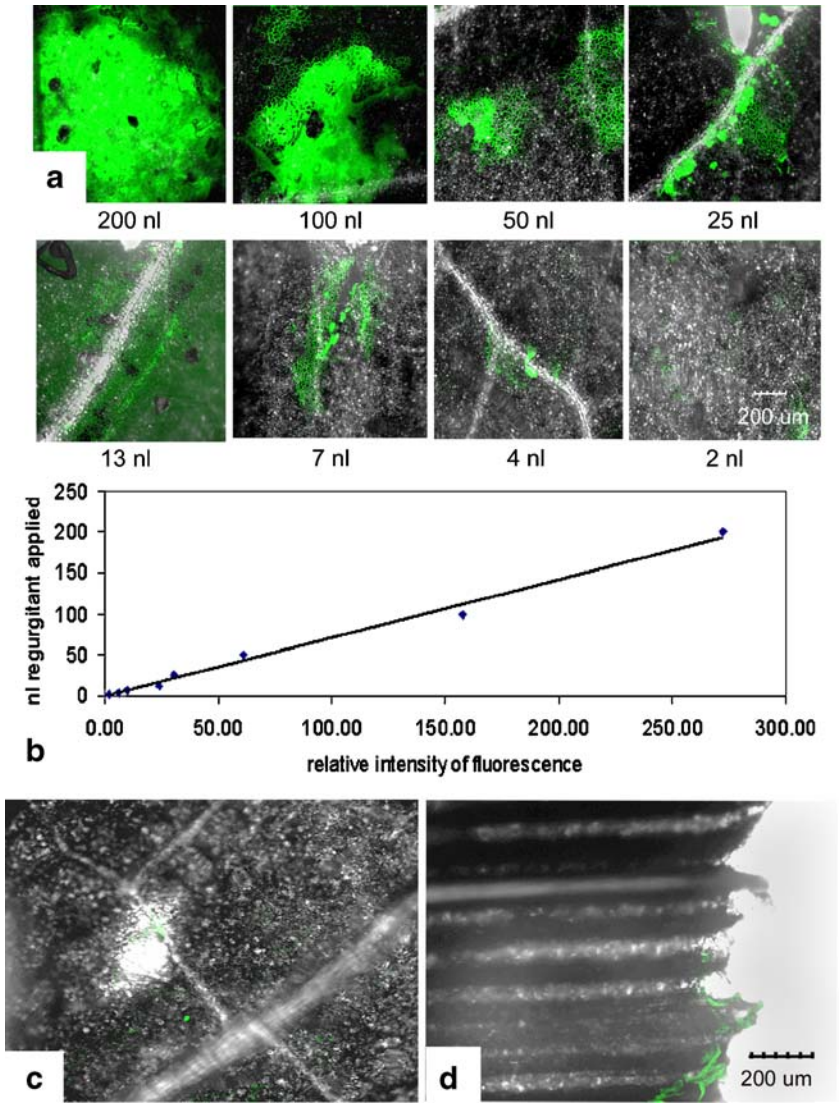

Fig. 3 Dilution series of regurgitant collected from *Helicoverpa zea* fed diet spiked with Alexa 488. **a** Regurgitant was diluted in water then 0.2 µl applied to tomato leaves; *numbers* represent the actual amount of regurgitant in the application. **b** The relative intensity increases exponentially with increasing amounts of regurgitant. R^2=0.992. Equation for the linear curve is y=0.7128x. **c** *H. zea* regurgitant at the feeding site of squash leaf from the linear curve the amount of regurgitant was calculated to be 0.27 nl. **d** *H. zea* regurgitant at the feeding site of corn leaf; from the linear curve, the amount of regurgitant was calculated to be 1.02 nl

regurgitant 24 h after being placed on the diet. Caterpillars were transferred to leaves and appeared to feed normally and, if agitated by the researcher, regurgitated easily. We were unable to detect any fluorescence in the caterpillar saliva or in the secretions of the ventral eversible gland of noctuid larvae, another secretory organ in this group of insects (Felton 2008). Thus, this technique specifically labels oral secretions or regurgitant in these species. By manually collecting regurgitant and applying it to leaves, we detected as little as 4 nl of regurgitant. Even with this sensitivity, however, only a fraction of the caterpillars left detectable regurgitant at the feeding site. Of those that did regurgitate, the amount was calculated to be ca. 0.5 to 5.0 nl per feeding bout. This is 4,000 to 40,000 times less than the 20 µl typically applied to plants. It is possible that some of the regurgitant is reingested during feeding, such that caterpillars are "eating their evidence" (Schittko et al. 2000). Although this cannot be ruled out by our methodology or that used by others (Truitt and Pare 2004), we suggest it would be unlikely that caterpillars would selectively regurgitate on foliage that was ingested and yet leave the feeding margins largely free of regurgitant. In the case of labial saliva, although some is reingested during feeding, substantial amounts of salivary glucose oxidase (microgram quantities) are left on feeding margins and also dropped on other portions of the leaf (Peiffer and Felton 2005).

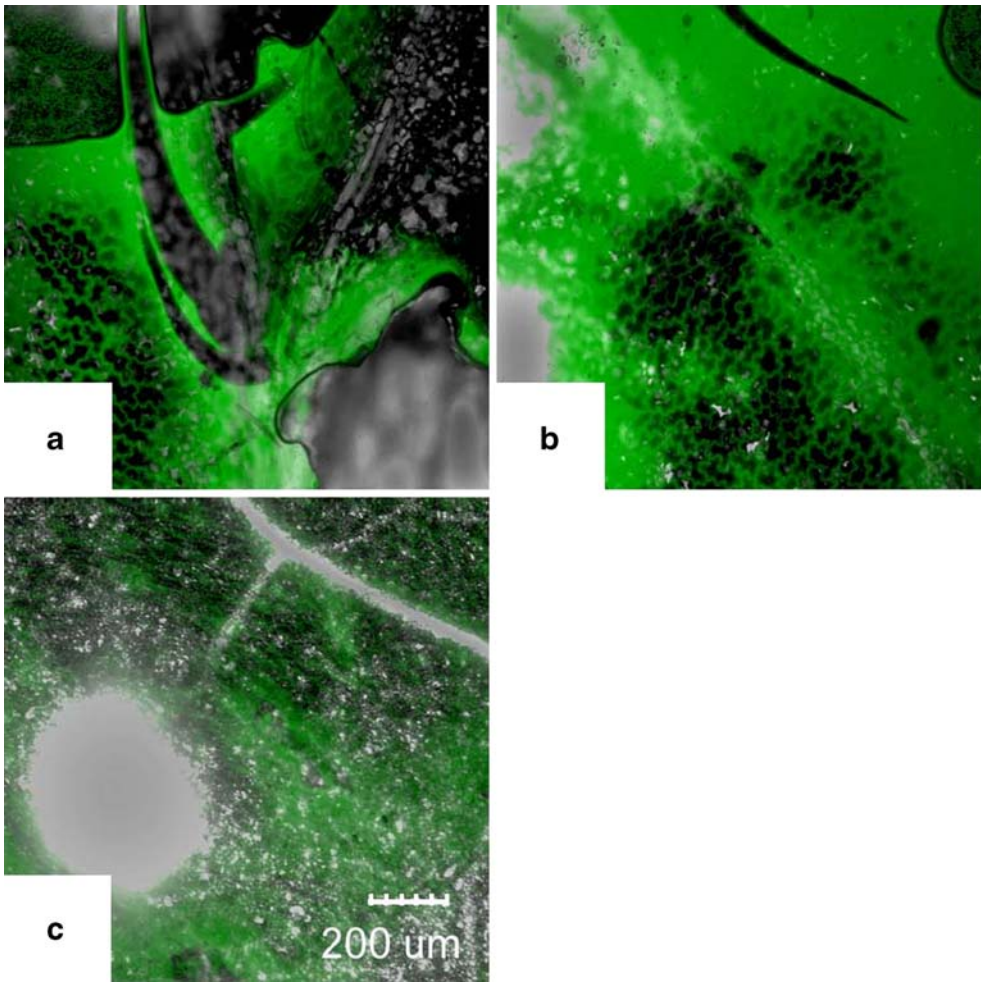

Fig. 4 Regurgitant from caterpillars fed diet spiked with Alexa 488 was collected and manually applied to tomato leaves after wounding with pattern wheel; **a** 1 μl of regurgitant from *Helicoverpa zea*. **b** 3 μl regurgitant from *H. zea*. **c** Regurgitant from *Manduca. sexta*, diluted 1:5, then 20 ul applied

Our data indicate that there are differences among caterpillar species in their predisposition to regurgitate during feeding. These differences may provide insight into how plants can differentiate feeding between related herbivore species, despite the species possessing similar elicitor profiles (De Moraes et al. 1998). The factors responsible for these differences in regurgitation during feeding are unknown, but differences in the propensity of caterpillar species to regurgitate in response to the threat of predation have been linked to the relative size of the fore- and midguts (Grant 2006). Those species with comparatively larger foreguts and smaller midguts are more prone to defensive regurgitation (Grant 2006). It should be noted that all of the caterpillar species used in our study have comparatively small foreguts (personal observations); thus, we do not know if there is also a similar constraint of gut structure and propensity to regurgitate during feeding, but this warrants further examination.

The propensity to regurgitate varied depending upon the host plant of the caterpillar. For instance, larval *H. zea* were far more likely to regurgitate when feeding on corn compared to other hosts such as tomato. The particular host factors that contribute to these differences are unknown but could be due to an array of factors associated with a particular host including water content, leaf toughness, nutritional status, and/or presence of specific toxins. Alternatively, it would be important to determine if caterpillars are able to specifically minimize regurgitation in order to prevent the elicitation of host defenses.

To date, few studies have included a dose-response analysis of OS when determining effects on induced plant responses. There have been an inexplicable number of studies that have used an identical quantity of OS for testing without regard to the species or stage of insect used. The fluorescent method developed here for specifically labeling OS provides the means to better estimate the actual amounts of OS that are released during feeding. Our results indicate that OS are not released during every caterpillar feeding bout, and they suggest that some herbivores may minimize their display of elicitors during feeding.

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Sex Pheromone of the Scarab Beetle *Phyllophaga* (*Phytalus*) *georgiana* (Horn)

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Abstract The sex pheromone of *Phyllophaga* (*Phytalus*) *georgiana* was characterized as valine methyl ester, tentatively the L-enantiomer. This is the first sex pheromone identified from the *Phyllophaga* subgenus *Phytalus*. The pheromone was extracted from female glands, the active component isolated by coupled gas chromatography–electroantennogram detection analysis, characterized by mass spectrometry, and shown to be active in field tests. The seasonal flight pattern was determined for *P. georgiana* as well as for three other species, *P. anxia* (both northern and southern genitalic forms), *P. gracilis*, and *P. postrema*. The latter three species were captured in traps baited with L-isoleucine methyl ester.

Keywords L-Valine methyl ester · L-Isoleucine methyl ester · Scarabaeidae · Melolonthinae · Cranberries

Sridhar Polavarapu, deceased May 7, 2004. We dedicate this publication to our friend and colleague.

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Introduction

The scarab beetle, *Phyllophaga* (*Phytalus*) *georgiana*, is distributed along the east coast of the United States from New Jersey to South Carolina, Georgia, Alabama, and Mississippi (Harpootlian 2001; Robbins et al. 2006). It is one of eight US species in the *Phyllophaga* subgenus *Phytalus*. The remaining *Phytalus* species (ca. 90) are distributed from Mexico, through Central America, to Brazil, Uruguay, and Paraguay (Evans and Smith 2007).

The larvae of *P. georgiana* are root-feeding pests of cranberries in New Jersey (Koppenhöfer et al. 2008). When the larvae were originally discovered feeding in the roots of cranberries, the species could not be identified because character differences in the larvae have yet to be described, although this is currently underway. To identify the species, ca. 50 larvae were reared to adulthood. The resulting adults were all identified as *P. georgiana.* We used these adults for the isolation and identification of the pheromone.

Materials and Methods

Pheromone Collections Larvae were dug from a bog in southern New Jersey and kept individually, in 30-ml plastic cups in a 3:1 mixture of greenhouse sand and screened peat moss raised to 12% moisture (*w*/*w*), in a controlled environment room, at 25°C for the 16-h photophase and

20°C for the 8-h scotophase. After eclosion, adult females were placed in observation cages to observe calling [abdominal pheromone gland everted (Leal et al. 1993; Nojima et al. 2003b)] during the scotophase. Once a female was observed calling, it was removed from the cage and its pheromone gland everted by applying gentle pressure to the abdomen. The gland was excised with scissors and extracted for 20 min in 200 μl of dichloromethane. After this, the gland was removed from the solvent and the extract was concentrated under a nitrogen stream to a volume of ca. 20 μl. Samples were stored at −80°C until analyzed.

Gas Chromatograph–Electroantennogram Detection Analysis Extracts of single glands were analyzed with a coupled gas chromatograph–electroantennogram detector (GC–EAD) system. A Hewlett Packard 5890 series II gas chromatograph, equipped with either a non-polar SPB-1 capillary column (30 m×0.25 mm ID, 0.25 μm film thickness; Supelco, Bellefonte, PA, USA) or a polar EC-WAX Econo-cap capillary column (30×0.25 mm ID, 0.25 μm film thickness; Alltech, Deerfield, IL, USA), was used for the GC–EAD analyses. The carrier gas was N_2 at a head pressure of 138 kPa (flow rate, 2.0 ml min^{-1}). Injections were made in the splitless mode (split valve opened at 1 min). The temperature program was 40°C for 5 min, then 15°C min^{-1} to 250°C. Injector, EAD outlet, and FID detector temperatures were 260°C. The column effluent was combined with N_2 make-up gas (30 ml min^{-1}) and split 1:1, between the flame ionization detector (FID) and EAD by using a TCD capillary column adapter (Agilent Technology, Wilmington, DE, USA), two deactivated capillary columns (non-polar, 0.25 mm ID, Alltech) of equal length as transfer lines, and a universal capillary Y connector (Alltech). The TCD adapter and the Y connector were connected with a short length of deactivated wide-bore capillary column (0.53 mm ID) and a stainless steel reducing union (3.2 mm OD to 1.6 mm OD). The end of the column was fixed to the TCD adapter with a regular column nut so that the column was extended to the inside of the wide-bore column to facilitate column changes.

An extra FID port of the GC was modified and used for the EAD outlet. The transfer line passed through the heating block and was protected and insulated with a short length of glass-lined stainless steel tube (1.6 mm OD× 0.3 mm ID, Alltech) and a larger diameter stainless steel tube. The transfer line terminated in a humidified, filtered air stream, which was refrigerated by a modified condenser flushed with ice-cold water. The air stream carried the column effluent over the beetle antennal preparation (Robbins et al. 2003). The output signal from the antenna was amplified by a customized single-step high-input impedance DC amplifier. The resulting signal was recorded on an HP 3390A integrator synchronized with the GC integrator. These methods were adapted from Zhang et al. (1997) and Nojima et al. (2003a). Gland extracts from ten virgin females were analyzed using antennae from five different males.

Chemical Analysis Gas chromatography–mass spectrometry (GC–MS) analysis was performed on a Shimadzu QP 5050A equipped with a non-polar DB-1ms capillary column (30 m×0.25 mm ID, 0.25 μm film thickness; J & W Scientific, Folsom, CA, USA) or a polar EC-WAX Econo-Cap capillary column (30 m×0.25 mm ID, 0.25 μm film thickness; Alltech), under the same conditions as those in the GC–EAD analyses, except helium was the carrier gas at 1.0 ml min^{-1}. The EAD-active compound was tentatively identified by comparing its mass spectrum to those of compounds in the NIST mass spectral library. Confirmation of the identification was made by comparing GC retention times (on the two columns) and mass spectrum of the beetle-produced product with those of the authentic sample.

Chemicals L-Valine and L-isoleucine methyl esters were supplied by Dr. A.C. Oehlschlager of ChemTica Internacional S.A. (San Jose, Costa Rica).

The D-valine methyl ester was obtained as a hydrochloric acid salt (Aldrich). Dissolving the salt in an aqueous alkali solution and extracting the mixture with three portions of diethyl ether generated the amino acid methyl ester. The ether extracts were combined, washed with brine, dried over sodium sulfate, and concentrated *in vacuo*. The resulting methyl ester was used without further purification.

Preparation and Field Evaluation of Synthetic Lures Lures were formulated by dissolving the neat compounds in hexane at 20 μg/μl and dispensing appropriate amounts in rubber septa (Thomas Scientific, Swedesboro, NJ, USA); the solvent was allowed to evaporate in a fume hood. Lures were deployed in the field in lab-constructed cross-vane traps (Robbins et al. 2006).

Six treatments were tested: L-valine methyl ester at 1, 2, and 4 mg; 4 mg L-isoleucine methyl ester; L-valine and L-isoleucine methyl esters at 4 mg each; and a control with hexane applied to the septum. L-Isoleucine methyl ester was included in this field trial because we suspected that it could function as a behavioral antagonist to *P. georgiana* when combined with L-valine methyl ester. Four replicates of the six treatments were deployed May 19, 2003, around the cranberry bog from which the *P. georgiana* larvae were sampled. The traps were placed about 15 m apart, with the bottom of the trap about 0.5 m from the ground. Traps were checked 18 times between May 19 and September 3. Traps were placed randomly at first placement and were re-randomized every other time they were checked. Lures were replaced every 4 weeks.

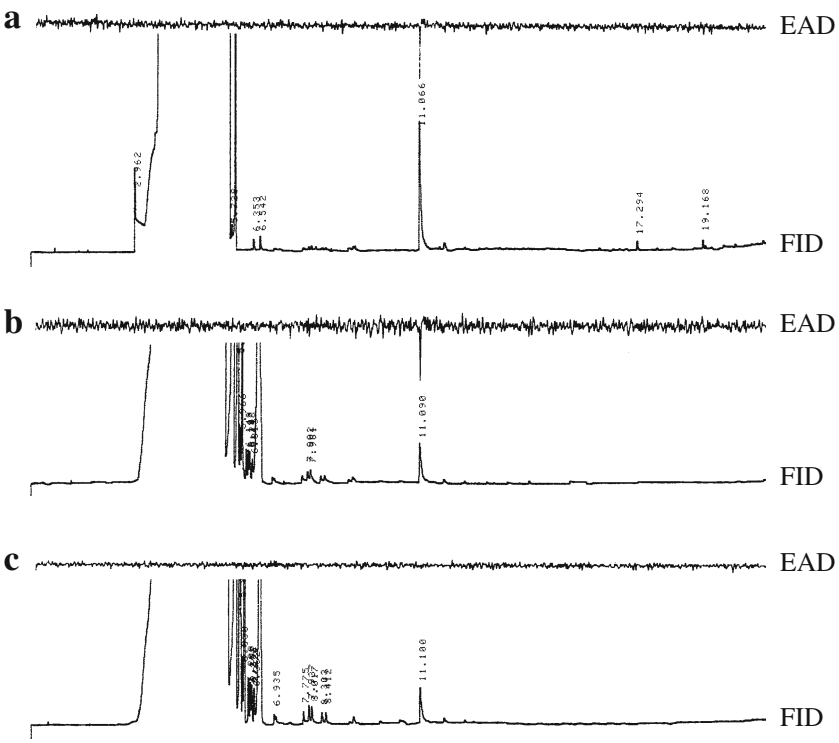

Fig. 1 Simultaneous electroantennogram (EAD)–flame ionization detection (FID) traces illustrating male *Phyllophaga georgiana* antennal responses to **a** extract of the pheromone gland of a female *P. georgiana*; **b** 10 ng of L-valine methyl ester; **c** 10 ng of D-valine methyl ester

On May 22, 2008, four traps were deployed at each of four cranberry bogs in southern New Jersey to confirm flight phenology for the beetle. Each trap was baited with a septum that contained 4 mg of L-valine methyl ester. The traps were placed at least 100 m apart, with the bottom about 0.5 m from the ground. Traps were checked 15 times between May 29 and September 10, and lures were replaced every 4 weeks. Traps were randomized at first placement and re-randomized every other time they were checked.

Captured males were identified to species according to Luginbill and Painter (1953) and Harpootlian (2001).

Statistics The *P. georgiana* trap catch data were tested for homogeneity of variance using Levene's test and then log-transformed ($x+1$). Data were analyzed using a one-way ANOVA, $P<0.05$. Fisher's LSD test was used for post hoc comparisons.

Results and Discussion

Pheromone Identification GC–EAD analysis, using both non-polar and polar columns, of gland extracts of female *P. georgiana* showed one consistent EAD-active compound (Fig. 1a). The mass spectrum of this compound matched that of L-valine methyl ester. GC retention times (on the two columns) compared with those of an authentic sample also confirmed the identification (Fig. 1b). The opposite enantiomer, D-valine methyl ester, did not elicit an EAD response from antennae of male beetles (Fig. 1c). A chiral column was not available at the time of analysis; therefore, we cannot be certain of the chirality of the valine methyl ester. However, based on the relative EAD activities (and field tests; see below), we tentatively assume the L-enantiomer to be the pheromone compound.

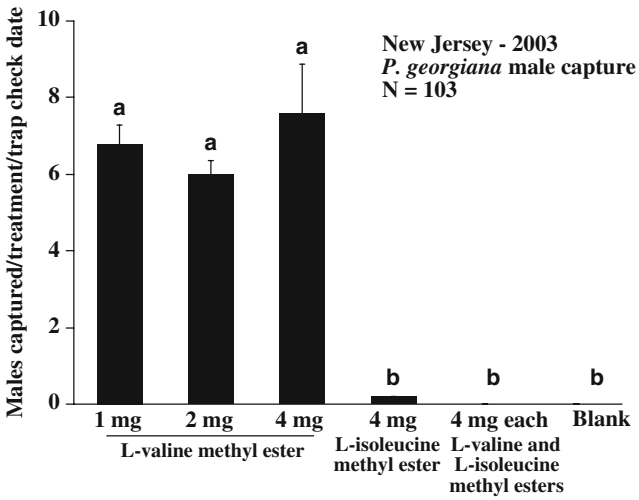

Fig. 2 Average capture/treatment/trap check (mean±SE) of male *Phyllophaga georgiana* in traps baited with various doses of L-valine methyl ester, L-isoleucine methyl ester, and a blend of L-valine/L-isoleucine methyl esters, at Chatsworth, NJ in 2003. *Bars with the same letter* are not significantly different ($P<0.05$, Fisher's LSD test)

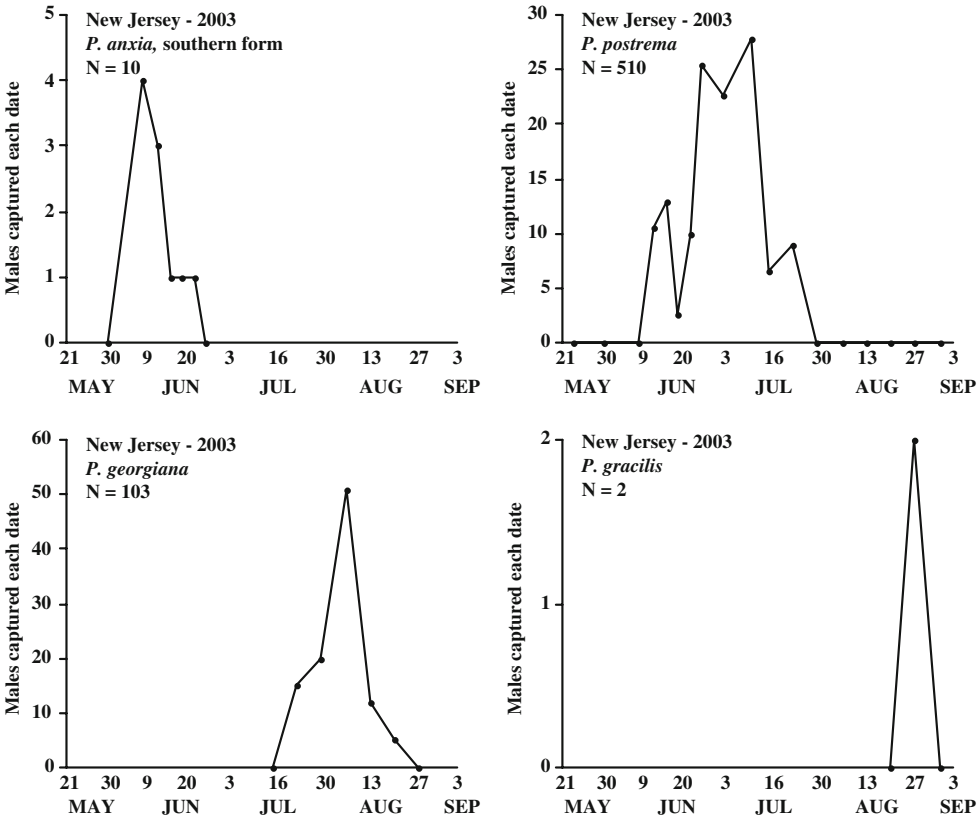

Fig. 3 Seasonal flight patterns and numbers of males of four species of *Phyllophaga* caught in pheromone-baited traps near Chatsworth, NJ in 2003

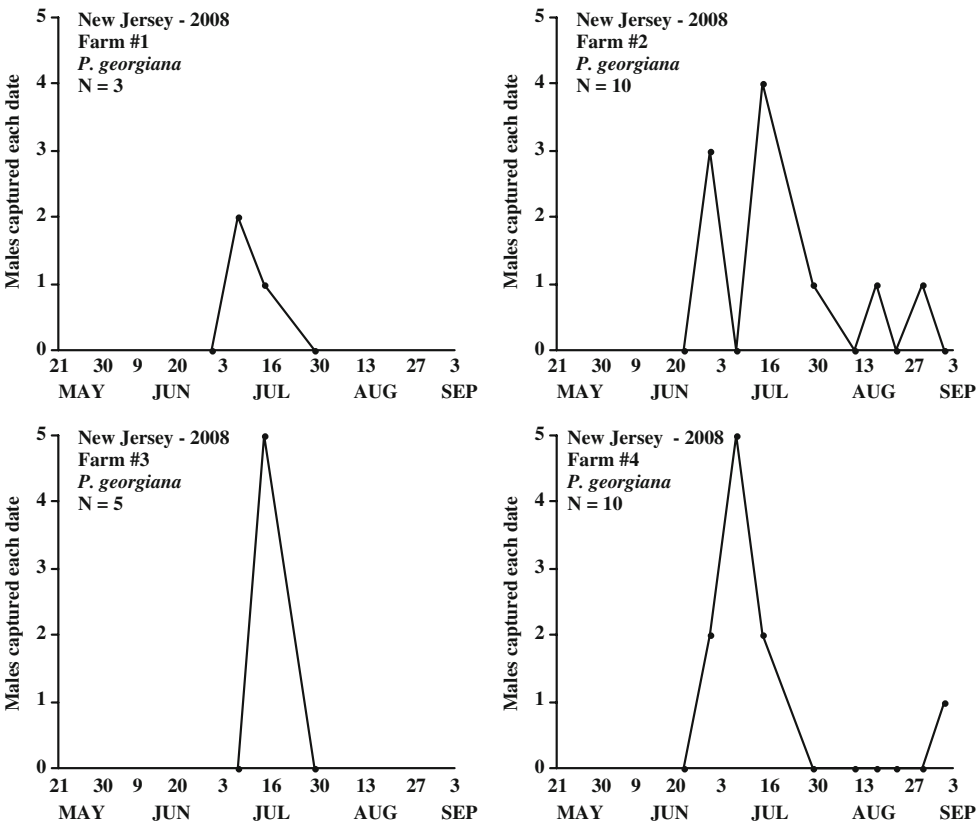

Fig. 4 Seasonal flight patterns and numbers of males of *Phyllophaga georgiana* captured in pheromone-baited traps at four farms near Chatsworth, NJ in 2008

Field Evaluation of Synthetic Lures One-way ANOVA indicated significant differences in *P. georgiana* male captures among the six treatments tested ($F_{5,24}$=7.70; $P<$ 0.001, Fig. 2). Fisher's LSD test in a post hoc comparison showed no significant differences among mean catches of the traps baited with 1, 2, or 4 mg of L-valine methyl ester. However, catches of males in traps baited with any of the three dosages of L-valine methyl ester were significantly greater than catches in traps baited with 4 mg L-isoleucine methyl ester-baited trap, 4 mg each of L-valine and L-isoleucine methyl esters, or the hexane control. A single *P. georgiana* male was captured in a trap baited with 4 mg of L-isoleucine methyl ester, and no males were captured in traps baited with 4 mg each of the methyl esters of L-valine and L-isoleucine, thus indicating that L-isoleucine methyl ester acts as a behavioral antagonist.

Our tentative identification of L-valine methyl ester as the sex pheromone of *P. georgiana* is consistent with previous observations in Tifton, GA in 2000 (Robbins et al. 2006). In that study, two male *P. georgiana* were captured in a trap baited with 4 mg of L-valine methyl ester on two dates in July 2000. The other seven traps at the Tifton location were baited with lures containing varying amounts of L-isoleucine methyl ester, from 10% to 100%, and no *P. georgiana* were captured in those traps.

Seasonal Flight Patterns for Species Captured Figure 3 illustrates the seasonal flight patterns and numbers of individuals of the four *Phyllophaga* species captured during the 2003 season. In general, the flight dates and male response to pheromone blends of the species observed in this test corroborate the results reported by Robbins et al. (2006).

Of the ten male *P. anxia* captured in traps baited with L-isoleucine methyl ester, eight were of the southern genitalic form and two were of the northern genitalic form [see Luginbill and Painter (1953) and Woodruff and Beck (1989)]. The southern forms were found on June 9 (four males), June 13 (3), and June 17 (1). The northern forms were found on dates following the flight of the southern forms, namely June 20 (1) and June 24 (1). Further studies may reveal whether the apparent asynchronous flights of the two L-isoleucine methyl ester-responding genitalic forms observed here are consistent over geography and years as well as whether interbreeding occurs in areas of sympatry. See Robbins et al. (2008) for more information regarding pheromone races of the northern and southern genitalic forms of *P. anxia*.

Of the 510 *P. postrema* captured between mid-June and mid-July, 506 were captured in traps baited with L-isoleucine methyl ester, and four were captured in traps baited with the blend of L-valine and L-isoleucine methyl esters. The two *P. gracilis* were captured on August 27 in traps baited with L-isoleucine methyl ester.

To date, there have been no studies on the life cycle of *P. georgiana*. However, the fact that the flight does not begin until mid-July suggests that this species probably pupates in the spring, unlike *P. anxia*, which pupates and emerges in the hibernaculum in the fall; the teneral adult remaining underground until the following May or June when the flights begin (Lim et al. 1980). The *P. georgiana* seasonal flight patterns observed at the four locations where traps were placed during the 2008 season (Fig. 4) were similar to the pattern observed in 2003 (Fig. 3).

Other species in the genus *Phyllophaga* (*s. str.*) also have been observed responding to L-valine methyl ester, including *P. anxia* (northern genitalic form, L-valine methyl ester pheromone morph), *P. congrua*, *P. crenulata*, *P. hirtiventris*, *P. inversa*, *P. latifrons*, *P. marginalis*, and *P. vehemens* (Robbins et al. 2006). Eberhard (1993) reported *Phyllophaga* males following pheromone upwind to non-conspecific females, landing on the female briefly, and then leaving. Further work should investigate the reproductive isolation of sympatric *Phyllophaga* species that respond to L-valine methyl ester.

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Sex Pheromones and Reproductive Isolation of Three Species in Genus *Adoxophyes*

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Abstract We tested differences in female pheromone production and male response in three species of the genus *Adoxophyes* in Korea. Females of all three species produced mixtures of (*Z*)-9-tetradecenyl acetate (*Z*9–14:OAc) and (*Z*)-11-tetradecenyl acetate (*Z*11–14:OAc) as major components but in quite different ratios. The ratio of *Z*9–14:OAc and *Z*11–14:OAc in pheromone gland extracts was estimated to be ca. 100:200 for *Adoxophyes honmai*, 100:25 for *Adoxophyes orana*, and 100:4,000 for *Adoxophyes* sp. Field tests showed that males of each species were preferentially attracted to the two-component blends of *Z*9–14:OAc and *Z*11–14:OAc mimicking the blends found in pheromone gland extracts of conspecific females. The effects of minor components identified in gland extracts on trap catches varied with species. Addition of 10-methyldodecyl acetate (10me-12:OAc) or (*E*)-11-tetradecenyl acetate (*E*11–14:OAc) to the binary blend of *Z*9–14:OAc and *Z*11–14:OAc significantly increased captures of *A. honmai* males, whereas *E*11–14:OAc exhibited a strongly antagonistic effect on catches of *Adoxophyes* sp. males. Moreover, (*Z*)-9-tetradecen-1-ol (*Z*9–14:OH) or (*Z*)-11-tetradecen-1-ol (*Z*11–14:OH) added to the binary blends increased attraction of male *A. orana* but not *A. honmai* and *Adoxophyes* sp. males, suggesting that these minor components, in addition to the relative ratios of the two major components, play an important role in reproductive isolation between *Adoxophyes* species in the southern and midwestern Korea where these species occur sympatrically.

Keywords *Adoxophyes* · (*Z*)-9-tetradecenyl acetate · (*Z*)-11-tetradecenyl acetate · Lepidoptera · Tortricidae · Reproductive isolation

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Introduction

Several species in the genus *Adoxophyes* (Lepidoptera: Tortricidae) are economically important pests of fruit and tea trees in Asia and Europe (Tamaki et al. 1971a; Meijer et al. 1971; Kou et al. 1990). In the past, it was believed that *Adoxophyes orana* is the only species found in Korea (Shin et al. 1994), but field trapping revealed that there is considerable variability in male response to pheromone blends among different geographical populations of *Adoxophyes* spp. (Han 2002; Yang et al. 2005). These findings prompted us to study the taxonomic status of Korean *Adoxophyes* populations by using mitochondrial gene sequences, and we found three distinct species, *Adoxophyes honmai*, *A. orana*, and *Adoxophyes* sp. (Park et al. 2008). Larvae are difficult to differentiate, but adults of the different species are distinguishable by wing patterns and male genitalia (Han 2002; Byun, personal communication).

Adoxophyes species often share host plants (Han 2002; Yang et al. 2005) and have some seasonal overlap in Korea (Choi 2002; Yang 2002). Furthermore, the diel periodicity of pheromonal emission also overlaps, with females of all

three species calling at the end of the scotophase and beginning of the photophase (Sato and Tamaki 1977; Den Otter and Klijnstra 1983; Han 2002). Therefore, differences in these parameters appear insufficient to maintain reproductive isolation among these species. Males of *Adoxophyes* species, however, are rarely attracted to heterospecific females in fields (Yang, unpublished), suggesting that species-specific blends of pheromone components may be responsible for reproductive isolation between sympatric species (Roelofs and Brown 1982; Löfstedt et al. 1991). Thus, the principal objectives of this study were to identify the sex pheromones of three *Adoxophyes* species and to determine the role of the pheromones as prezygotic reproductive isolating mechanisms between sympatric congeners in Korea.

Methods and Materials

Insects

Larvae of *Adoxophyes* spp. were collected from pear orchards in Naju, Cheonan, and Namyangju, Korea during June and July of 2006. The larvae were reared individually on artificial diet (Bio-serv, Frenchtown, NJ, USA) in transparent plastic Petri dishes (1.5 cm high and 5.5 cm diameter) and maintained at 23°C under a 16:8-h ratio of light to dark photoperiod. Pupae were separated by sex and kept individually in plastic bottles (7 cm high and 2.5 cm diameter). After eclosion, moths were identified based on forewing color patterns (Han 2002) and then provided with a cotton pad soaked with a 5% (*w*/*v*) sucrose solution as food.

Chemicals

Synthetic dodecenyl acetate (12:OAc), tridecyl acetate (13:OAc), (*Z*)-9-dodecenyl acetate (*Z*9–12:OAc), 10-methyldodecyl acetate (10me-12:OAc), tetradecyl acetate (14:OAc), *Z*9–14:OAc, *E*11–14:OAc, *Z*11–14:OAc, (*Z*)-11-hexadecenyl acetate (*Z*11–16:OAc), (*Z*)-9-tetradecen-1-ol (*Z*9–14:OH), (*Z*)-11-tetradecen-1-ol (*Z*11–14:OH), and octadecanal (18:Ald) were purchased from Pherobank (Wageningen, The Netherlands). Isomeric purity of these compounds exceeded 99%.

Gland Extraction

Pheromone gland extracts were taken from 2- to 3-day-old females at the end of the scotophase, during their calling periods (Den Otter and Klijnstra 1983; Han 2002). The pheromone gland of each female was extruded by applying gentle pressure to the abdomen, excised with fine forceps, and individually extracted in 10 μl hexane containing 10 ng of 13:OAc as an internal standard in a 0.3-ml conical vial (Wheaton, Millville, NJ, USA) for 30 min at room temperature. The supernatant was transferred into another vial and stored at −80°C until analysis.

Chemical Analysis

Pheromone gland extracts were analyzed on an Agilent 6890N GC equipped with a split/splitless injector and a flame ionization detector. Samples were run on DB-5 and DB-Wax columns (30 m×0.25 mm ID, 0.25 μm film thickness, J&W Scientific, Folsom, CA, USA) in the splitless mode. Injector and detector temperature were 250°C. Helium was used as carrier gas (1 ml/min). The gas chromatograph (GC) oven temperature was programmed from 80°C (1 min hold) to 220°C at 5°C/min and held for 10 min. Components in pheromone gland extracts were identified by comparison of retention times with those of authentic standards on two different columns. The quantity of each component was estimated by comparing its GC peak area with that of the internal standard.

Gas chromatography–mass spectrometry (MS) analyses of the crude extract of pheromone glands were performed on an Agilent 6890N GC interfaced to an Agilent 5973 mass-selective detector. Samples were analyzed on the DB-Wax column (30 m×0.25 mm ID, 0.25 μm film thickness) with the temperature program described above. The ionization voltage was 70 eV. The ion source temperature was 230°C. Components in gland extracts were tentatively identified by comparison of their mass spectra with the mass spectra library (Wiley-NIST, Hoboken, NJ, USA), and identifications were confirmed by comparison of retention times and mass spectra with those of authentic standards.

Field Experiments

Field experiments were conducted in pear orchards at Naju, Cheonan, and Namyangju, respectively, during May–July of 2007. Sticky Delta traps (Green Agro Tech, Korea) baited with rubber septa (Aldrich Chemical Co., Milwaukee, WI, USA) impregnated with test chemicals in hexane were hung on branches 1–1.5 m above ground level. All field tests employed a complete randomized block design with five replicate blocks. The distance between traps within a block was at least 15 m. Twice a week, moths were counted, removed, and identified to species by using wing venation and then verified by examination of the genitalia.

Experiment 1 investigated the attraction of *A. honmai*, *A. orana*, and *Adoxophyes* sp. males to the two major components *Z*9–14:OAc and *Z*11–14:OAc singly and in binary blends. Experiment 2 was conducted to test the individual effects of the nine minor components identified

in female gland extracts; 12:OAc, *Z*9–12:OAc, 10me-12:OAc, 14:OAc, *E*11–14:OAc, *Z*9–14:OH, *Z*11–14:OH, *Z*11–16:OAc, and 18:Ald as possible synergists or antagonists, by using standard baits of the two major components. The standard baits used for *A. honmai*, *A. orana*, and *Adoxophyes* sp. were 1 mg of 35:65, 80:20, and 3:97 mixtures of *Z*9–14:OAc and *Z*11–14:OAc, respectively. Trap catch data (x) were transformed to log (x+1) and submitted to one-way analysis of variance. Means were compared by Tukey's test at α=0.05 (SAS Institute Inc. 2004).

Distribution of *Adoxophyes* Species

We sampled larvae of *Adoxophyes* spp. from pear orchards at eight different sites in Korea during June–August of 2007 and 2008 and reared them on artificial diet. After eclosion, female moths were assigned to three species according to their wing characters and subsequently verified by examination of the sex pheromones. Individuals were classified as *A. honmai*, *A. orana*, and *Adoxophyes* sp. when *Z*9–14:OAc and *Z*11–14:OAc in pheromone gland extracts were present in a ca. 100:200, 100:25, and 100:4,000 ratios, respectively. In all cases, the pheromone blend of individual glands supported the initial species identification based on adult morphological characters.

Results

Chemical Analysis

GC and GC–MS analyses of pheromone gland extracts showed that *Z*11–14:OAc is the major component of *A. honmai* and *Adoxophyes* sp., whereas *Z*9–14:OAc is the most abundant component in the pheromone blend of *A. orana* (Fig. 1). The ratios of *Z*9–14:OAc and *Z*11–14:OAc in pheromone blends were 100:209, 100:25, and 100:3,810 for *A. honmai*, *A. orana*, and *Adoxophyes* sp., respectively. In addition to these two major components, three acetate esters (12:OAc, 14:OAc, and *E*11–14:OAc), *Z*11–14:OH, and 18:Ald, as well as nonadecane, heneicosane, and tricosane, were detected in gland extracts of all species (Table 1). Small amounts of *Z*9–14:OH and *Z*11–16:OAc were detected in *A. honmai* and *A. orana*, while *Z*9–12:OAc and 10me-12:OAc were present only in *A. honmai*.

Field Experiments

Traps baited with *Z*9–14:OAc or *Z*11–14:OAc alone caught no *Adoxophyes* males. Responses of males to various blends of *Z*9–14:OAc and *Z*11–14:OAc differed markedly among species. Male *A. honmai* were equally attracted

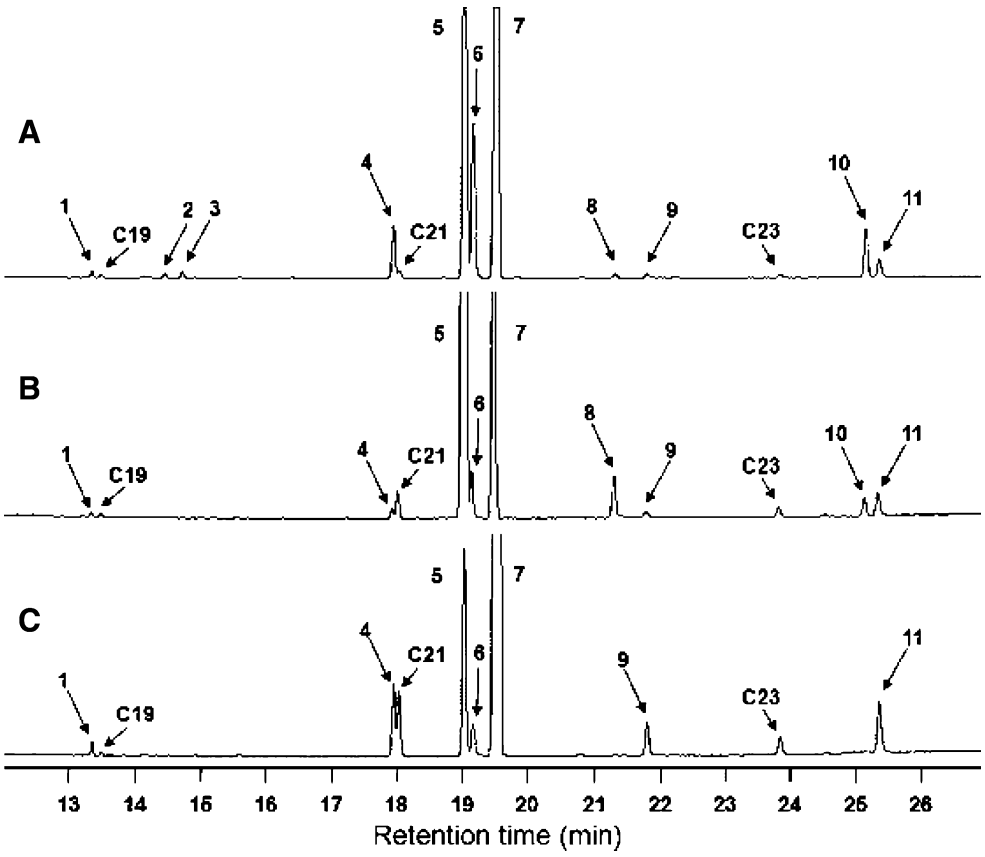

Fig. 1 Comparison of gas chromatograms of pheromone gland extracts of *Adoxophyes honmai* (*A*), *A. orana* (*B*), and *Adoxophyes* sp. (*C*) collected in Korea. *1* Dodecyl acetate, *2* (*Z*)-9-dodecenyl acetate, *3* 10-methyldodecyl acetate, *4* tetradecyl acetate, *5* (*Z*)-9-tetradecenyl acetate, *6* (*E*)-11-tetradecenyl acetate, *7* (*Z*)-11-tetradecenyl acetate, *8* (*Z*)-9-tetradecen-1-ol, *9* (*Z*)-11-tetradecen-1-ol, *10* (*Z*)-11-hexadecenyl acetate, *11* octadecanal, *C19* nonadecane, *C21* heneicosane, *C23* tricosane

Table 1 The amount (ng) and relative ratios (mean ± SE) of pheromone components in female extracts of three Korean *Adoxophyes* species (N=25)

Compound	*A. honmai*		*A. orana*		*Adoxophyes* sp.	
	Amount	Ratio	Amount	Ratio	Amount	Ratio
Dodecyl acetate	0.29±0.05	0.8±0.1	0.23±0.04	0.8±0.2	0.16±0.02	7.1±0.7
(*Z*)-9-dodecenyl acetate	0.25±0.04	0.7±0.1	–	–	–	–
10-Methyldodecyl acetate	0.32±0.06	0.9±0.2	–	–	–	–
Tetradecyl acetate	2.44±0.30	6.9±0.6	0.60±0.13	1.7±0.4	0.80±0.09	31.9±2.6
(*Z*)-9-tetradecenyl acetate	35.88±4.18	100	38.38±3.15	100	2.65±0.27	100
(*E*)-11-tetradecenyl acetate	5.02±0.58	14.7±1.6	0.54±0.07	1.6±0.2	0.40±0.04	16.9±1.3
(*Z*)-11-tetradecenyl acetate	73.77±8.19	209±6.3	10.05±1.26	25.4±1.4	98.44±9.62	3,810±245
(*Z*)-9-tetradecen-1-ol	0.25±0.02	0.8±0.1	0.32±0.05	0.9±0.1	–	–
(*Z*)-11-tetradecen-1-ol	0.41±0.03	1.3±0.1	0.11±0.01	0.3±0.1	0.31±0.06	11.6±1.8
(*Z*)-11-hexadecenyl acetate	1.65±0.27	4.6±0.7	0.68±0.13	2.0±0.6	–	–
Octadecanal	1.28±0.12	4.3±0.4	0.82±0.14	2.5±0.5	0.90±0.11	39.7±4.9

30:70 to 80:20 ratios of *Z*9–14:OAc and *Z*11–14:OAc (Fig. 2a), while *A. orana* males were preferentially attracted to ratios from 50:50 to 90:10 (Fig. 2b). In contrast, *Adoxophyes* sp. males were most attracted to traps baited with *Z*11–14:OAc as a major component (Fig. 2c). Regression analysis indicated a positive linear relationship between trap catch of male *A. orana* and *Z*9–14:OAc concentration for binary blends (r^2=0.75, P<0.05), while a negative relationship was observed with *Adoxophyes* sp. (r^2=0.86, P<0.01).

The effects of minor component of gland extracts on field trap catches varied significantly among species. Specifically 10me-12:OAc and *E*11–14:OAc showed a strong synergistic effect with the 35:65 mixture of *Z*9–14:OAc and *Z*11–14:OAc for *A. honmai* (Fig. 3a), while the capture of male *A. orana* increased when the two alcohols,

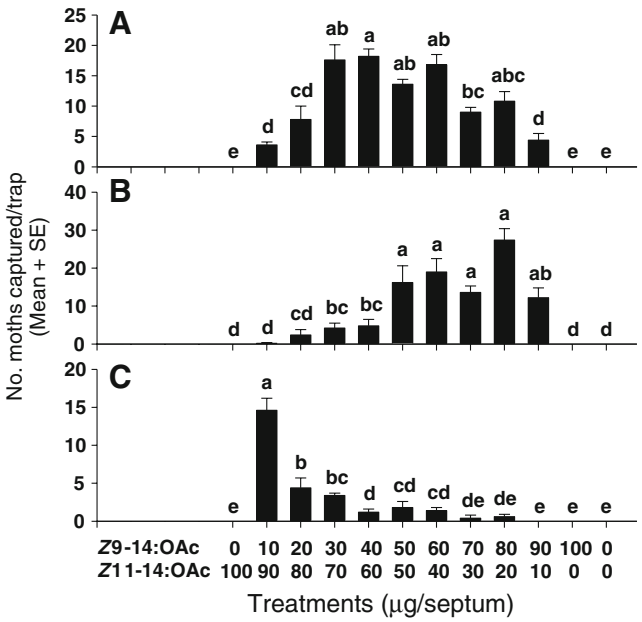

Fig. 2 Number of *A. honmai* (**a**; Naju, May 11–18, 2007), *A. orana* (**b**; Cheonan, May 17–24, 2007), and *Adoxophyes* sp. (**c**; Namyangju, May 18–25, 2007) males captured in traps baited with lures containing different ratios of *Z*9–14:OAc and *Z*11–14:OAc at pear orchards in Korea. *Bars with the same letter* are not significantly different (Tukey's test: P>0.05)

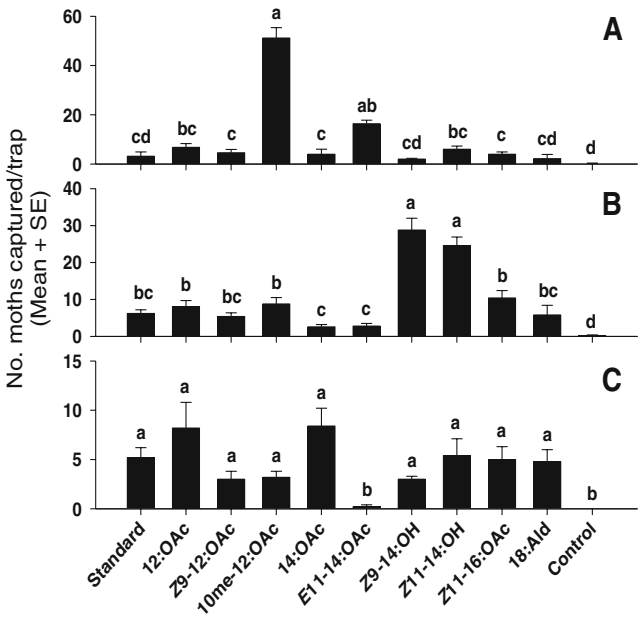

Fig. 3 The effect of adding 5% of different minor components to standard baits with 1 mg/septum of *Z*9–14:OAc and *Z*11–14:OAc on captures of male *A. honmai* (**a**; Naju, June 22–29, 2007), *A. orana* (**b**; Cheonan, July 12–19, 2007), and *Adoxophyes* sp. (**c**; Namyangju, July 13–20, 2007) at pear orchards in Korea. *Bars with the same letter* are not significantly different (Tukey's test: P>0.05)

*Z*9–14:OH and *Z*11–14:OH, were added to the 80:20 mixture of *Z*9–14:OAc and *Z*11–14:OAc (Fig. 3b). In the case of *Adoxophyes* sp., the only significant effect of minor components was the strong inhibitory effect of adding *E*11–14:OAc to a 3:97 ratio of the primary binary blend (Fig. 3c).

Distribution of *Adoxophyes* Species

It is evident from the pheromone gland analyses of females collected as larvae from different sites that there is considerable difference in the geographic distribution of the three species (Table 2).

Discussion

The analyses of glands extracts indicate that all three *Adoxophyes* species have *Z*9–14:OAc and *Z*11–14:OAc as major components, but in markedly different ratios. Males were preferentially attracted to the blend found in pheromone gland extracts of their conspecific females, but males of *A. honmai* and *A. orana* both responded to a broad range of ratios of *Z*9–14:OAc and *Z*11–14:OAc. These findings suggest that while difference in the relative composition of two major pheromone components is a component in the reproductive isolation of the sympatric *Adoxophyes* species, this is not sufficient to provide species-specific communication channels. However, our data suggest that two minor components, 10me-12:OAc and *E*11–14:OAc, play a role in isolating *A. honmai* and *Adoxophyes* sp. where they occur sympatrically in the southern part (Ulju, Jinju) of Korea.

A. orana also produces *Z*9–14:OH and *Z*11–14:OH. Their presence in the lure increases trap catch and our results support the idea that these two alcohols, as well as the relative ratios of the two major components, contribute to the maintenance of premating reproductive isolation between sympatric populations of *A. orana* and *Adoxophyes* sp. in the midwestern part (Ansung, Cheonan) of Korea. In our study, we found no evidence that *A. honmai* and *A. orana* occur sympatrically in Korea, but if they do, the differences in the ratios of major components and synergistic pheromone compounds, such as 10me-12:OAc and *Z*9–14:OH, may prevent cross-attraction between them.

Even though *E*11–14:OAc is present at low levels in the pheromone glands of *A. orana* and *Adoxophyes* sp., the addition of this compound to standard baits significantly decreased attraction for conspecific males. The antagonistic effect of minor compounds found in gland extracts of conspecific females has been reported for other moths (McElfresh and Millar 1999; Wu et al. 1999). However, it is not known whether *E*11–14:OAc is actually released from the female glands during calling. Further research is required to compare the attractiveness of pheromone blends actually emitted with those found in female gland extracts of these two species. Similarly, the roles of *Z*9–12:OAc and *Z*11–16:OAc, present in small amounts in gland extracts of some *Adoxophyes* species, need to be elucidated. Do they function as antagonists to sympatric moth species, or are they just by-products of the pheromone biosynthesis? Additionally, the influence of several components present simultaneously within blends on male trap catch also needs to be investigated.

Female pheromone glands from Japanese females of *A. honmai* contain *Z*9–14:OAc and *Z*11–14:OAc as major components at 67:33 (Tamaki et al. 1971a) or 73:27 (Noguchi et al. 1985) ratios, with two behaviorally important minor components, 10me-12:OAc and *E*11–14:OAc, at 4–5% and 2–3% of the two major components, respectively (Tamaki et al. 1979; Noguchi et al. 1985). These ratios are markedly different from those observed in this study (see Table 1), providing evidence of pheromonal variation in geographic populations of *A. honmai*. Interestingly, the observed ratio of the two major pheromone components in Korean *A. honmai* feeding on tea tree as well as on fruit trees (Han 2002) is similar to that reported

Table 2 The relative proportion of three *Adoxophyes* species collected from pear orchards at eight different sites in Korea

Site	Geographic location	*A. honmai*		*A. orana*		*Adoxophyes* sp.	
		2007	2008	2007	2008	2007	2008
Namyangju	37.4° N, 127.1° E	0	0	0	0	37	15
Ansung	37.0° N, 127.1° E	0	0	2	5	7	17
Cheonan	36.5° N, 127.1° E	0	0	42	25	11	8
Yeongi	36.4° N, 127.2° E	0	0	0	0	5	9
Sangju	36.2° N, 128.1° E	0	0	0	0	22	16
Ulju	35.2° N, 129.2° E	10	17	0	0	4	8
Jinju	35.1° N, 128.1° E	21	14	0	0	5	2
Naju	35.0° N, 126.4° E	45	22	0	0	0	0

from an *Adoxophyes* sp. from tea gardens in Taiwan (Kou et al. 1990), again supporting the idea of geographic variability in the pheromones within the genus.

The pheromone glands of *A. orana* females in Switzerland (Guerin et al. 1986) and females from different populations of *A. orana fasciata* in Japan (Tamaki et al. 1971b; Sugie et al. 1984; Noguchi et al. 1985) have similar ratios (77:23 to 88:12) of the two major components. Furthermore, pheromone traps baited with lures with similar ratios are effective in capturing males at all sites (Tamaki et al. 1971b; Meijer et al. 1971; Sugie et al. 1984; Guerin et al. 1986), thus supporting the idea that populations of *A. orana* and/or *A. orana fasciata* in Asia and Europe use similar blends of the major pheromone components. The addition of *Z*9–14:OH or *Z*11–14:OH to the binary blend of *Z*9–14:OAc and *Z*11–14:OAc significantly increased captures of *A. orana* males in Europe (Guerin et al. 1986), and a similar pattern was noted in our field trials in Korea (Fig. 3). However, while these minor components are present in pheromone gland extracts of *A. orana fasciata* (Noguchi et al. 1985), their addition to lures does not enhance trap efficiency in Japan (Sugie et al. 1984). Thus, the pheromone characteristics of Korean population of *A. orana* are identical to those of European population of *A. orana*, but different from those reported for Japanese *A. orana fasciata*.

Our field results show that *Adoxophyes* sp. is the dominant species of *Adoxophyes* in Korea. This species has a 3:97 blend of *Z*9–14:OAc and *Z*11–14:OAc, a ratio that has not been reported from any *Adoxophyes* species in Japan, Taiwan, or Europe. A 1:9 ratio of *Z*9–14:OAc and *Z*11–14:OAc captured *Adoxophyes privatana* males in Vietnam (Hai et al. 2002), so the Korean *Adoxophyes* sp. may be *A. privatana*, although nothing is known about the pheromone gland content of this species. Additional research that examines different *Adoxophyes* species around the world is necessary to clarify the composition of sex pheromones of different species within this genus. Parallel morphological and molecular studies should also be carried out to clarify the phylogenetic relationships and to understand the evolution of pheromone communication in this genus.

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Sex Pheromone of the Bud Borer *Epinotia aporema*: Chemical Identification and Male Behavioral Response

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Abstract *Epinotia aporema* (Walsingham) is a Neotropical pest of legumes in southern South America. Its importance has increased during the last decade owing to the significant growth of soybean production in the region. Monitoring of *E. aporema* is difficult due to the cryptic behavior of the larvae, and hence, chemical control is carried out preventively. We analyzed the female-produced sex pheromone so as to develop monitoring traps and explore pheromone-based control methods. We analyzed pheromone gland extracts by combined chromatographic, spectrometric, and electrophysiological methods. Based on the comparison of retention indices, mass spectra, and electroantennogram (EAD) activity of the insect-produced compounds with those of synthetic standards, we identified two EAD-active compounds, (*Z*,*Z*)-7,9-dodecadienol and (*Z*,*Z*)-7,9-dodecadienyl acetate (15:1 ratio), as sex pheromone components of *E. aporema*. We also studied the behavior of males in wind tunnel tests using virgin females and different combinations of synthetic standards (15:1, 1:1, and 1:0 alcohol/acetate) as stimuli. A significantly greater percentage of males reached the chemical source with the 15:1 synthetic mixture than with any of the other treatments, indicating that these two compounds are pheromone components.

Keywords Sex pheromones · *Epinotia aporema* · Tortricidae · Wind tunnel · Lepidoptera

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Introduction

Soybean production in southern South America has increased dramatically in recent years. In Uruguay, for example, the planted area has expanded 30-fold during the period 2000–2007, causing a tenfold increase in the use of insecticides such as endosulfan and chlorpyrifos (Ferrari 2007). During the same period, the area of soybean production in Brazil grew to almost 60%, reaching more than 22 million hectares (Conab 2007).

Epinotia aporema (Walsingham, 1914) (Lepidoptera: Tortricidae) is a stem and bud borer that has become a major pest of legumes in Uruguay, Argentina, Chile, and southern Brazil (Iede and Foerster 1982; Sanchez et al. 1997; Pereyra and Sanchez 1998; Alzugaray et al. 1999). *E. aporema* originates from Costa Rica, but is now widely distributed throughout South America. The insect has five to six generations per year, mostly concentrated between November and April (Bentancourt and Scatoni 2006).

The economic importance of *E. aporema* is restricted to the south of its distribution due to the year-round availability of hosts (soybean, alfalfa, lotus, peas, flax, red clover) (Biezanko et al. 1957; Morey 1972; Bentancourt and Scatoni 1995; Alzugaray and Ribeiro 2000). In soybean, the larvae feed on vegetative plant parts, affecting the normal growth of the plant. In other legumes, larval feeding can severely affect the flower and prevent production of seeds, an important commodity in some forage

legumes (alfalfa, lotus) (Bentancourt and Scatoni 1995; Alzugaray and Ribeiro 2000).

E. aporema populations are monitored by direct observation of the larvae, a highly inefficient method with poor predictive capacity, and chemical control is, therefore, used in a prophylactic way. In order to develop a monitoring tool for the insect, we studied its sex pheromone. In this study, we report the characterization of the sex pheromone of *E. aporema* as a 15:1 mixture of (*Z*,*Z*)-7,9-dodecadienol and (*Z*,*Z*)-7,9-dodecadienyl acetate (hereafter referred to as Z7,Z9-12:OH and Z7,Z9-12:Ac, respectively).

Methods and Materials

Insects *E. aporema* adults were obtained from a laboratory population kept in an incubator, under a 14:10-h (L/D) photoregime, 21–23°C, and 60–70% relative humidity. The larvae were raised on an artificial diet based on bean homogenate and agar, either in glass tubes or in plastic cups. Virgin adults were obtained by separating male and female pupae.

Gland Extractions Female pheromone glands were dissected between the fifth and seventh hours of the scotophase. Virgin females (2–6 days old) were placed individually in glass tubes (10 cm length, 8 cm diameter; gauze-closed) during the photophase. Following the scotophase, calling females were removed from the vials, killed, and their glands exposed and excised with forceps. The glands of 83 females were accumulated in a 0.2-mL conical vial that was stored at −15°C. Pooled glands were extracted with 100 μL of distilled hexane for 20 min at ambient temperature, and the extract concentrated under a stream of N_2.

Chemical and Gas Chromatography–Electroantennogram Detection Analyses Gas chromatography–electroantennogram detection (GC-EAD) analyses were carried out using an HP 5890 Series II Gas Chromatograph adapted for simultaneous flame ionization (FID)/EAD detection. The column effluent was split with a fused silica outlet splitter (Alltech) with an extra make-up flow of N_2 (30 mL min^{-1}) added before the splitter. The column directing the flow to the EAD left the oven upwards into a condenser inner tube (1 cm diameter) where a stream of charcoal-filtered and humidified air (flow 300 mL min^{-1}) transported the volatile chemicals to the antennal preparation. The water in the condenser was kept cold by a circulating chiller. The antenna was located 1 cm downstream from the column exit and was held between antenna holders (Syntech, Hilversum, The Netherlands) with electrically conductive gel. The holders were in turn connected to a preamplifying probe (10X, Syntech), and the EAD response was further amplified with a high-impedance amplifier (Syntech). The analog signal was finally fed back to the GC motherboard for digitization and processing.

The GC-EAD was equipped with an Elite-WAX or an Elite-5 column (Perkin Elmer) (30 m×0.25 mm i.d., 0.25 μm film thickness), operated with a constant carrier gas flow of 2 mL min^{-1} (H_2). The temperature program for both columns was 70°C (kept for 1 min) to 250°C at 7°C min^{-1}. Injection was splitless with injector and detector temperatures of 220°C and 250°C, respectively.

GC–mass spectrometry (MS) analyses were carried out on a QP-2010 Shimadzu GC-MS, equipped with an AT™-WAX MS column (Alltech) (30 m×0.25 mm, 0.25 μm), operated with a constant carrier flow of 1 mL min^{-1} (He) and splitless injection. The initial temperature was 70°C (held 1 min), then increased to 250°C at 8°C min^{-1}. The injector temperature was 220°C, and the interface temperature was 250°C.

Retention indices (RIs) of the EAD-active components were determined by using the GC-EAD system by coinjecting a hexane solution of *n*-alkanes (even-numbered C8–C24, plus C19, 100 ppm each) with the gland extracts. RIs were calculated as defined for programmed temperature analysis (arithmetic index in Adams 2007).

Synthetic standards of the acetates were kindly provided by Tetsu Ando (Tokyo University of Agriculture and Technology) and Christer Löfstedt (Lund University). The alcohol standards were obtained by hydrolysis of the corresponding acetates (10% NaOH in ethanol, 100°C, 1 h).

Wind Tunnel Experiments The responses of males to virgin calling females and mixtures of synthetic standards were evaluated. The wind tunnel consisted of a cylindrical acrylic glass tube (150 cm length; 40 cm diameter) with a controlled suction pump, activated charcoal filters, and a metal screen to laminate the airflow (0.3 m s^{-1}, measured with a Testo 490 anemometer). Environmental conditions during wind tunnel tests were 20–25°C and 50–70% relative humidity. Dimmed red light was used for the observations. Males used in the experiments came from the laboratory colony. Four hours before testing, 1- to 5-day-old virgin males were placed in individual glass tubes (8 cm length, 4.5 cm diameter) with gauze enclosing both ends.

The stimuli (virgin females or pheromone septa) were hung at the tunnel entrance, centered, and inside a glass tube (10 cm length, 8 cm diameter; gauze-enclosed for the females). For the virgin female test, five females (2–5 days old) were placed in the tunnel 4 h before the experiments; for each male tested, at least one of the females was observed calling throughout the test period. Z7,Z9-12:OH and Z7,Z9-12:Ac (at mixtures of 15:1, 1:1, and 1:0 alcohol/acetate) were loaded (in 10 μL hexane) onto red rubber sleeve septa with a total dose of 1.6 μg per septum. Control septa were loaded

with 10 μL hexane. Septa were changed every day of the test with totals of ten males tested to each pheromone blend and 28 to the virgin females.

The tests were run between the third and seventh hours of the scotophase. Each tube containing a male was hung at the end of the tunnel, centered, and left closed for 1 min to allow the pheromone plume to reach the male. The gauze was then removed from both ends of the tube, and the behavior of the male was recorded continuously for 10 min. The behavioral elements observed were the time to initiate flight, response to the pheromone (including flying within the plume and/or wing fluttering), and reaching the odor source. Behavioral data were analyzed using analysis of variance and Dunnett's pairwise comparisons for the time of flight initiation [transformed to log (X+1)], and chi-square 2×2 contingency tables to compare the proportions of males arriving at the source and responding to the pheromone.

Results and Discussion

Chemical and GC-EAD Analysis Hexane extracts of pheromone glands [one female equivalent injected in 1 μL] elicited two consistent EAD responses from the male antennae. Only one of the EAD-active compounds was observed as a faint FID peak, while the other was only observed in the EAD trace (Fig. 1a; EAD responses shown with injection of synthetic compounds in Fig. 1b). Coinjection of the gland extracts and a mixture of *n*-alkanes in the GC-EAD system allowed us to calculate the RIs of both EAD-active compounds in the gland extract (2,076 and 2,155 on an Elite-WAX column; 1,529 and 1,664 on Elite-5). In addition, the GC-EAD analyses on both GC phases showed that the two EAD-active compounds inverted their relative elution order with the major compound eluting second on Elite-WAX (RI=2159) and first on Elite-5 (RI=1529). This inversion and the 135-unit difference in the RIs on Elite-5 suggested the presence of an alcohol/acetate pair in the mixture with alcohol as the major component. Moreover, the RI on both GC phases was around the region of RIs known for conjugated dodecadienyl alcohols (e.g., Δ5,Δ7: range 1,511–1,537 on DB5; 2,161–2,197 on DB-wax) and acetates (e.g., Δ5,Δ7: ranges 1,636–1,666 on DB5; 2,063–2,108 on DB-wax) (El-Sayed 2008). Previously, a 1:1 mixture of Z7,Z9-12:Ac and Z7,Z9-12:OH had been identified in the female gland of a European congener, *E. tedella* (Priesner et al. 1989), which prompted us to compare our pheromone extract with synthetic standards of these compounds. The standards were injected as a 100-ppm mixture in the GC-EAD system, giving strong EAD responses from *E. aporema* male antennae with identical retention times to the compounds in female extract that elicited the EAD signals (Fig. 1b).

GC-MS analysis of a concentrated (ten female equivalents per microliter) female gland extract revealed the presence of two peaks with identical retention times and mass spectra to those of Z7,Z9-12:Ac and Z7,Z9-12:OH standards (Fig. 2). The alcohol (both natural and synthetic) showed a clear M^+ peak (*m*/*z* 182) with a loss of 18 mass units and a weak fragment at *m*/*z* 31, both typical of primary alcohols. Both spectra showed the characteristic ions of aliphatic dienes (*m*/*z* 67, 82, and 96) and high abundance of the M^+ ion which, along with the long retention time on Elite-WAX (96 RI units larger than a

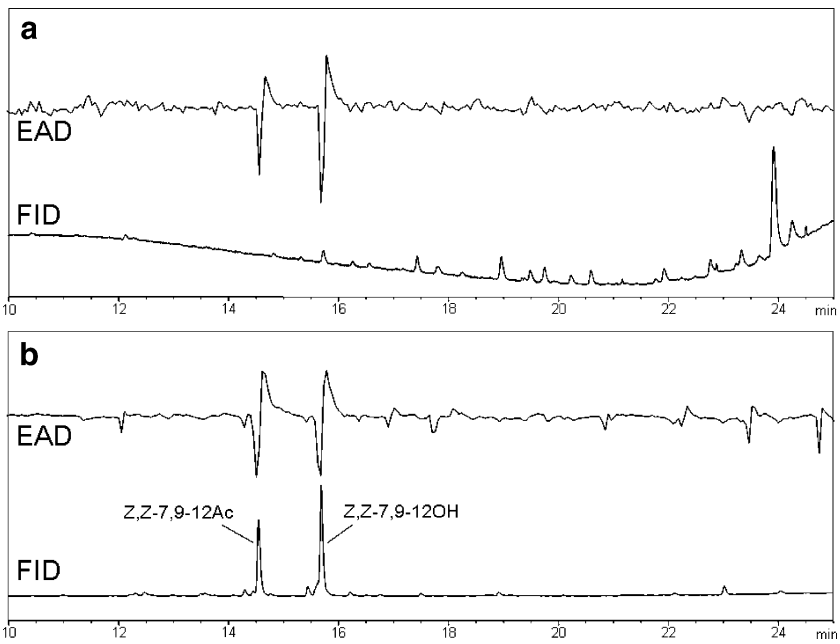

Fig. 1 Coupled flame ionization (*FID*)–electroantennogram detection (*EAD*) of **a** one female equivalent of *E. aporema* pheromone gland extract and **b** (*Z*,*Z*)-7,9-dodecadienyl acetate (*Z7,Z9-12:Ac*) and (*Z*,*Z*)-7,9-dodecadienol (*Z7,Z9-12:OH*) synthetic standards (100 ppm solution). Runs were performed consecutively on an Elite-5 column, each with a fresh male antenna, using identical temperature programs and splitless injection

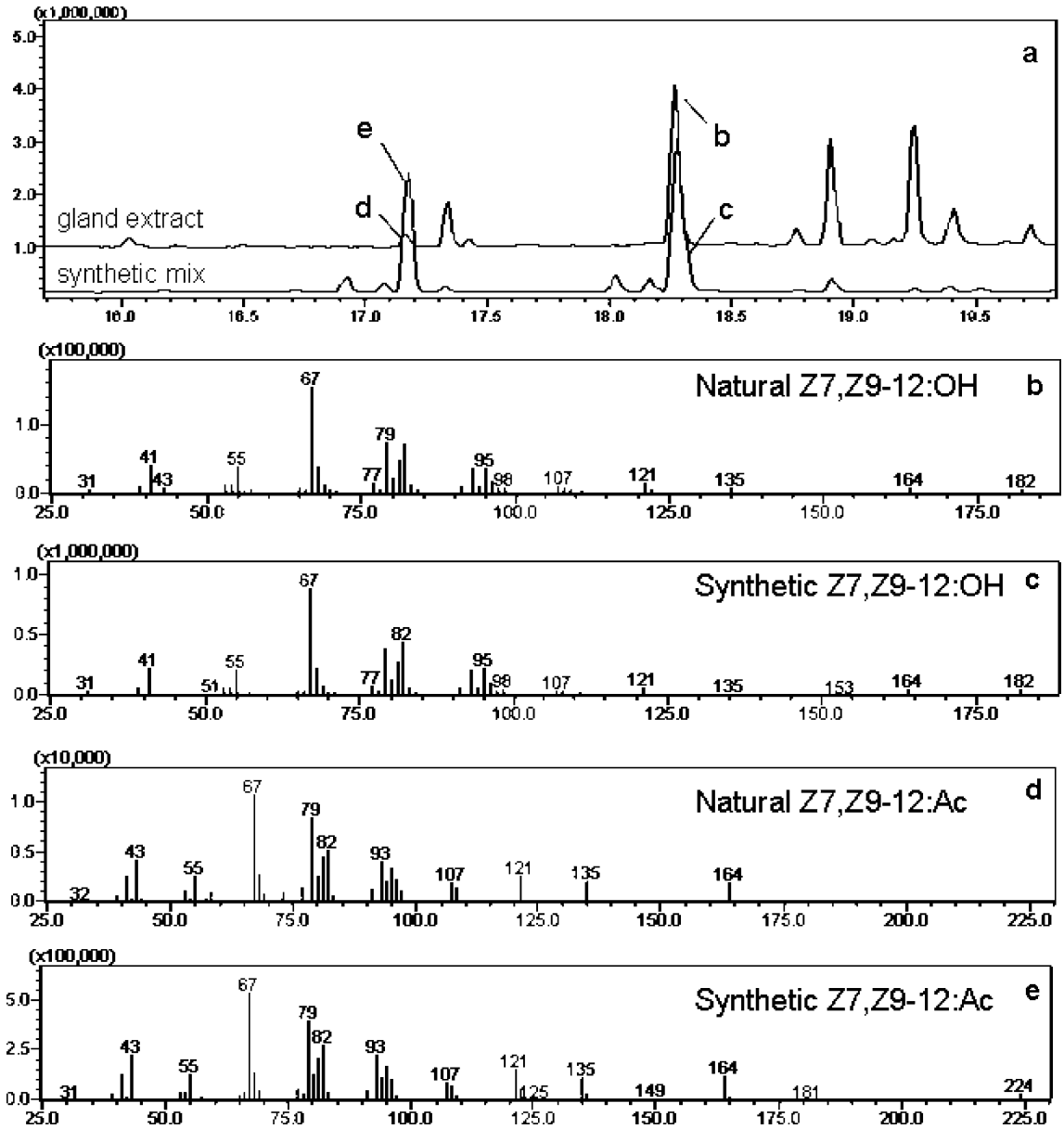

Fig. 2 Comparison of GC-MS analyses of ten female equivalent extracts of *E. aporema* pheromone glands (**a**, *upper trace*) and a synthetic mixture of (*Z*,*Z*)-7,9-dodecadienyl acetate (*Z7,Z9-12:Ac*) and (*Z*,*Z*)-7,9-dodecadienol (*Z7,Z9-12:OH*) (**a**, *lower trace*). The mass spectra of natural (**b**, **d**) and synthetic (**c**, **e**) compounds are shown. Runs were performed consecutively on an AT-WAX column, using identical temperature programs and splitless injection

nonconjugated dodecadienyl alcohol; El-Sayed 2008), indicated a conjugated dienyl system (Fig. 2b, c) (Ando et al. 2004). The natural and synthetic acetates showed almost identical spectra (Fig. 2d, e). The fragments characteristic of aliphatic dienes were also present with an additional fragment at *m*/*z* 43 (CH_3CO^+) and a molecular ion at *m*/*z* 224 in the synthetic sample. The absence of the molecular ion in the spectrum of the natural acetate is attributed to the low amount of the compound in the sample. The total ion chromatogram of gland extracts showed that the alcohol and the acetate were present in a 15:1 ratio.

A second set of comparative GC-MS analyses was performed with the pheromone gland extract and mixtures of geometric isomers of 7,9-dodecadienol and 7,9-dodecadienyl acetate. The retention time of the natural 7,9-dodecadienol matched that of synthetic Z7,Z9-12:OH (see Supplementary Figure S1), while the retention time of the natural Z7,Z9-12:Ac (Fig. 2d) matched that of synthetic Z7,Z9-12:Ac. There was an unknown compound in the gland extract that eluted at the same time as the E7, E9-12:Ac, but its mass spectrum (not shown) indicated that it was not a 7,9-12:Ac isomer.

These two compounds are produced by the European congener, *E. tedella*, in a 1:1 ratio (Priesner et al. 1989). In fact, most genera within the tribe Eucosmini (subfamily: Olethreutinae) include species that are attracted to Δ7 and/or Δ9 dodecenyl/dodecadienyl alcohols and acetates (El-Sayed 2008). Therefore, this Neotropical tortricid produces compounds closely related chemically to its closest relatives.

Wind Tunnel Experiments The time for male flight initiation ranged between a few seconds up to 3 min. The shortest time corresponded to the responses to virgin females, which was significantly shorter than the flight initiation time to the control. The tests with the synthetic pheromone blends resulted in intermediate flight initiation times, which were not significantly different from times to either virgin females or the control (Fig. 3a). In terms of response to the pheromone (including flying within the plume and/or wing fluttering), the synthetic pheromone blend with the same proportion as found in the female glands (15:1), the pure alcohol (1:0), and virgin females elicited the greatest responses with 70%, 60%, and 50% of males, respectively, responding to these treatments. However, only the 15:1 synthetic blend elicited a significantly greater percentage of males to respond than did the control and 1:1 (i.e., high proportion of acetate) treatments (Fig. 3b).

With respect to the arrival at the pheromone source, the 15:1 synthetic blend was the most attractive with 60% of males arriving at the septum within 10 min. In most cases, males arrived, left, and flew back to the source several times. The percentage of males arriving at the source for this treatment was significantly greater than for all other treatments. Less than 15% of males arrived at the calling females or the septa loaded with other blends, and no males arrived at the control septa (Fig. 3c).

Although the 15:1 proportion of the two pheromone components in the gland extracts may not reflect the actual proportion emitted by the female, the synthetic treatment with this ratio elicited the strongest responses from males, even at the low dose at which it was tested (1.6 μg). Although males responded to the alcohol-only treatment, indicating that this compound is probably crucial for male responses, the lower rates of males arriving at the source (compared to the 15:1 blend) indicates that the acetate is also an important component of the blend. The relatively low responses (arriving, especially) of males to the virgin females may be due either to an atypical or discontinuous pheromone emission because of the stressful conditions of the wind tunnel tests or simply to the low amount of pheromone females emit compared to that from the septa.

Pheromone-based monitoring of *E. aporema* could become an important tool for the integrated management of this pest in soybean crops in southern South America.

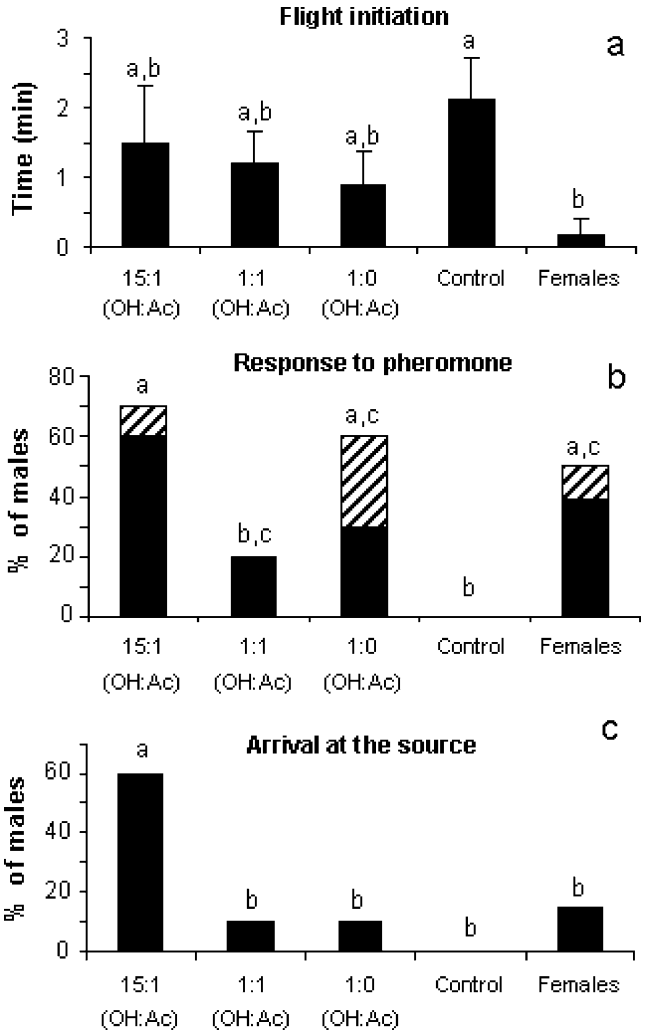

Fig. 3 Behavioral responses of *E. aporema* males to various treatments including virgin calling females and rubber septa loaded with different blends of (*Z*,*Z*)-7,9-dodecadienyl acetate (*Ac*) and (*Z*,*Z*)-7,9-dodecadienol (*OH*). **a** Time for flight initiation (mean minutes±SEM). **b** Percentage of males responding to the pheromone (*solid bars* flight within the plume, *striped bars* wing fluttering only). **c** Percentage of males arriving at the pheromone source. *Different letters above treatments* indicate significant differences at $P<0.05$

Control of this pest is complicated by the fact that the larvae are inside buds. Hence, monitoring of adults could be used to schedule insecticide applications more precisely, when larvae are exposed. The deficiency of a monitoring system not only results in overuse of insecticide, but also hinders the development of alternative control measures, such as use of insect hormone mimics or biological control. These alternatives need to be used within a specific time window during larval development, which is only possible after accurate detection of adult flight. Moreover, insecticides for the control of *E. aporema* are usually employed early in the growing season, causing a decline in populations of natural enemies that would otherwise control later-appearing noctuid moths. This, in turn, results in more

insecticides being applied later in the season; a trend that, combined with the expansion of soybean production, is threatening the sustainability of soybean and neighboring agroecosystems. To evaluate the potential of pheromone-based monitoring of *E. aporema*, field tests with various blends and pheromone doses are planned for the upcoming austral summer season.

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Fine Tuning of Social Integration by Two Myrmecophiles of the Ponerine Army Ant, *Leptogenys distinguenda*

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Abstract Myrmecophiles are animals that live in close association with ants and that frequently develop elaborate mechanisms to infiltrate their well-defended host societies. We compare the social integration strategies of two myrmecophilic species, the spider, *Gamasomorpha maschwitzi*, and the newly described silverfish, *Malayatelura ponerophila* gen. n. sp. n., into colonies of the ponerine army ant, *Leptogenys distinguenda* (Emery) (Hymenoptera: Formicidae). Both symbionts use chemical mimicry through adoption of host cuticular hydrocarbons. Exchange experiments between *L. distinguenda* and an undetermined *Leptogenys* species demonstrate that reduced aggression toward alien ants and increased social acceptance occurred with individuals of higher chemical similarity in their cuticular hydrocarbon profiles. We found striking differences in chemical and behavioral strategies between the two myrmecophiles. Spider cuticular hydrocarbon profiles were chemically less similar to the host than silverfish profiles were. Nevertheless, spiders received significantly fewer attacks from host ants and survived longer in laboratory colonies, whereas silverfish were treated with high aggression and were killed more frequently. When discovered and confronted by the host, silverfish tended to escape and were chased aggressively, whereas spiders remained in contact with the confronting host ant until aggression ceased. Thus, spiders relied less on chemical mimicry but were nevertheless accepted more frequently by the host on the basis of behavioral mechanisms. These findings give insights into the fine tuning of social integration mechanisms and show the significance of qualitative differences among strategies.

Keywords Ants · Chemical mimicry · Cuticular hydrocarbons · *Gamasomorpha maschwitzi* · Hymenoptera · Formicidae · *Malayatelura ponerophila* · Myrmecophiles · Nestmate recognition · Silverfish · Social integration · Spiders

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Introduction

Ant societies may function as small ecosystems for various other animals (Hölldobler and Wilson 1990). Especially large ant colonies offer ecological niches free of enemies and rich in food resources for organisms that have managed to intrude into the host's social system. Although such symbionts frequently partake in host resources, the term symbiosis originally did not imply a particular positive or negative interaction between the partners (Goff 1982).

Symbionts that live in close association with ants are also frequently referred to as myrmecophiles. To attain social integration, myrmecophiles apply adaptive strategies of appeasing or deterring the host or of circumventing the nest recognition system (Lenoir et al. 2001). Specifically with migratory ants, myrmecophiles require additional mechanisms of maintaining contact with their mobile hosts. Although many army ants impose such challenges by being highly migratory, forming only temporary nests (bivouacs) that are shifted frequently, they exhibit an extraordinarily diverse fauna of myrmecophiles (Gotwald 1995). This is probably due to the large colonies that offer numerous ecological niches and to the availability of considerable amounts of high-quality food that is retrieved during regular and highly efficient raiding activities. The Southeast Asian ponerine, *Leptogenys distinguenda* (Emery) (Hymenoptera: Formicidae), which reaches colony sizes of more than 50,000 workers and exhibits army ant behavior (Maschwitz et al. 1989), is also inhabited by numerous myrmecophiles, including beetles, phorid flies, springtails, silverfish, woodlice, spiders, and snails (Witte 2001; Witte et al. 2008). By depleting colony resources or by direct predation on the host, myrmecophiles might exert fitness costs on their host (Kistner 1982), and under these circumstances, selection may have occurred such that the host now displays counterstrategies to minimize the costs of symbionts. In this study, we analyzed the specific interactions for two selected myrmecophiles and their host, *L. distinguenda*, in order to identify potential host countermeasures that would reduce symbiont pressure. We compared the integration levels of the oonopid spider, *Gamasomorpha maschwitzi* (Wunderlich 1994), and the newly described silverfish, *Malayatelura ponerophila* gen. n. sp. n. (Mendes et al., unpublished). Both of these symbionts occur in most *L. distinguenda* colonies, and since they consume some of the host's resources, they are costly for the host (Witte et al. 2008). In this study, we compared the chemical and behavioral integration strategies of these two morphologically and systematically different symbiont species.

Chemical cues are known to play a major role in social insect recognition mechanisms; social insects generally use characteristic hydrocarbon profiles on the cuticle to recognize nestmates (Reese 1982; Vender Meer et al. 1982; Howard and Blomquist 2005; Hefetz 2007). These surface chemicals are transferred constantly among nestmates, passively by physical contact and actively by grooming and trophallaxis (Soroker et al. 1995). Myrmecophiles have been found to mimic the chemical profiles of their hosts either by biosynthesis of cuticular hydrocarbons or by acquiring the host chemical profiles passively (Dettner and Liepert 1994). The term "chemical mimicry" is used in this paper according to the original definition of the term "mimicry," as one organism's resemblance to another organism's properties (Vane-Wright 1976). Since chemical profiles of ant colonies may change considerably over time (Hölldobler and Wilson 1990), intruders must be able to update their profiles constantly in order to avoid detection. We hypothesize that myrmecophiles will fail to match host profiles if they do not continuously renew their chemical cues. At some point, profiles will diverge to such an extent that hosts can detect, expel, or even kill myrmecophiles. Once a certain state of mismatch in recognition cues has been reached, it must become increasingly difficult to approach host workers and to update the chemical profiles for organisms relying on passive chemical mimicry. By negative feedback, this process might even lead to complete social rejection, which would be fatal for obligate symbionts. Hence, symbionts might pay high costs for the exploitation of their host. We show in this study that considerable differences exist in the social acceptance of two common symbionts and that their social integration is a well-balanced and potentially fragile system.

Methods and Materials

We used a fourfold approach to study the behavioral and chemical integration of the myrmecophiles into the colony of their host: (1) studies of behavioral interactions between host ants and myrmecophiles in two colonies, which included their native myrmecophiles, but were reduced in size to enable laboratory observations; (2) studies of recognition and aggression between colonies with different cuticular hydrocarbon profiles; (3) identification of cuticular hydrocarbons in free-living host colonies based on the samples of highest concentrations; and (4) analyses of chemical similarities between hosts and myrmecophiles based on a large dataset of relative quantities of the principal cuticular hydrocarbon components from six free-living colonies.

Behavioral Observations We studied the host ant *L. distinguenda* in a well-recovered secondary, dipterocarp, lowland rainforest ecosystem at the Field Studies Centre of the University Malaya in Ulu Gombak, Malaysia (03°19.4796′ N, 101°45.1630′ E; elevation, 228.8 m). Studies were carried out for a total of 9 weeks, 3 weeks each in March and September 2006 and another 3 weeks in March 2007. Host colonies were located during their activity phase at night by retracing raiding columns to the nest sites. Nest sites were numbered, marked with tape, and checked every 30 min between 9:00 P.M. and 3:00 A.M. for ongoing activities. Army ants shift their nest sites frequently, carrying all larvae and pupae in a continuous column to a new location (migration). With *L. distinguenda*, such migrations last several hours. When a colony was found emigrating, *G. maschwitzi* and *M. ponerophila* were collected with aspirators directly from the emigration column. Adult and young teneral adult host

workers (also known as callows), larvae, and pupae also were collected to assemble simplified observation nests for behavioral studies (two colonies) and for exchange experiments (two additional colonies) at the field station (see below). Six additional collections, again from different free-living host colonies, were subjected to cuticular hydrocarbon extraction and chemical analyses (see below). To permit investigation of potential influences of nest composition on the interactions between the host and each symbiont, laboratory colonies were assembled with a different demographic composition. Worker numbers ranged from 83 to 140, callows from 3 to 34, larvae from 15 to 28, and pupae from 9 to 53. Ant colonies were housed in clear plastic containers (1×14×20 cm) with a 1-cm wide entrance, shaded with cartridge covers, and placed upside down on a moistened plaster floor in an arena (9×25×32 cm). Arenas were covered with plastic lids when no observations were carried out, and the side walls were treated with paraffin oil to prevent escape of the ants. These nests were for behavioral studies only, so the paraffin treatment did not affect our chemical analysis.

Interactions among spiders, silverfish, and host ants were monitored during nightly observation sessions of defined length, on average, 12 sessions of 5 min per night. Observation times of the four laboratory nests summed up to more than 38 hours and included 20 spiders and 41 silverfish. Observations were carried out both before and after feeding. Each day before the observation sessions, total numbers of all nest inhabitants were counted. For statistical analysis, the behaviors observed between the host and the spider and between the host and the silverfish were standardized in a behavioral index (F_r). This was necessary to compare laboratory nests of different composition, considering that the odds of interactions are influenced by the number of potentially interacting individuals and the observation time. The index (F_r) standardizes the frequency of a particular behavioral pattern between the objects A and B relative to the number of individuals in the focal groups (A and B) and observation time with $F_r = [F_o/(N_A \times N_B \times t)]$, where F_o is the observed frequency, N_A is the number of animals in group A, N_B is the number of animals in group B, and t is the observation time. Behavioral indices were evaluated with nonparametric statistical methods (Wilcoxon-matched pairs test, Mann–Whitney U test, and Kruskal–Wallis test). Furthermore, the data were standardized to 100% for each symbiont species to compare the overall proportions of behavioral patterns.

To study the dependency of social acceptance on colony-specific chemical cues, reciprocal exchange experiments were carried out between two *L. distinguenda* laboratory colonies, including 40 host ants, ten spiders, and three silverfish. Unfortunately, spiders and silverfish could be exchanged only in smaller numbers due to their overall rarity, their low social acceptance, and particularly the mortality of silverfish from aggressive behavior by the host. Twenty ants of a closely related (undescribed) *Leptogenys* species also were tested as a control. This species resembled *L. distinguenda* morphologically and showed similar behavior, including army ant mass raiding. Specimens of this species were deposited at the Ludwig-Maximilians Universität (LMU, Munich, Germany). For intercolonial exchanges, individuals from one colony were captured by using a padded aspirator and introduced carefully into a different observation colony without causing disturbance. All interactions were monitored for the following 10 min and forward jerking toward an introduced animal; chasing, mandible grabbing, snapping, biting, or stinging were all classified as "aggression." All kinds of direct physical contact between two focal objects were considered as "contact." In addition to these behavioral observations, cuticular hydrocarbons were extracted as described below from the experimental colonies as well as the control species.

Chemical Analyses We examined the cuticular hydrocarbon profiles of the *Leptogenys* colonies that were used for laboratory exchange experiments to correlate observed aggression levels directly with chemical similarities/dissimilarities. In addition, chemical analyses were conducted with six *L. distinguenda* field colonies including their spider and silverfish symbionts to unravel the chemical resemblance between symbionts and their hosts in natural colonies.

Cuticular hydrocarbons were extracted by placing individual insects (or spiders) in 2-ml vials with polytetrafluoroethylene septa and treated for 5 min with 200 μl of pentane (HPLC grade, Sigma-Aldrich). The crude extracts containing both polar and non-polar components were analyzed at the LMU by coupled gas chromatography and mass spectrometry (GC-MS) on an Agilent Technologies 6890N GC and 5975 MSD (70 eV, EI) equipped with a Restek Rxi-5MS column (30 m length, 0.25 mm i.d., 0.25-μm film thickness). Injections were performed in the pulsed splitless mode over 1.0 min at 280°C, including an initial pressure pulse of 16 psi for 0.5 min, followed by automatic flow control at 1.0 ml/min with helium as the carrier gas. The oven program began isothermally at 120°C for 2 min, then increased at 25°C/min until 200°C was reached, followed by a temperature ramp of 4°C/min until the final temperature of 300°C was reached. The transfer line was held constantly at 310°C. A range of 50–500 amu was scanned after an initial solvent delay of 3.8 min.

Identification of Compounds In the most concentrated extracts, individual compounds, mostly hydrocarbons, were identified by their mass spectra, their retention indices, and comparison with library spectra (Wiley7N). As far as possible, concentrations were evaluated semiquantitatively according to the relative contribution of each peak to the total peak area of the sample. Structures of saturated unbranched and branched hydrocarbons were assigned according to well-

established procedures (Carlson et al. 1998; Schulz 2001). Double-bond positions in alkenes and alkadienes were identified by derivatization of the crude extract with dimethyldisulfide (DMDS; Francis and Veland 1981; Vincenti et al. 1987; Carlson et al. 1989; Schulz and Nishida 1996). The resulting adducts allowed assignment of the (*E*)-or (*Z*)-configuration only in cases when both diastereomers were present in the extract. Because of the completely stereoselective reaction of DMDS, the adduct derived from the (*Z*)-compound eluted before the (*E*)-adduct on apolar gas chromatographic phases (Leonhardt and Devilbiss 1985). Double-bond positions in tri-, tetra-, and pentanes were identified tentatively by analysis of mass spectra and rationalization of fragmentation patterns according to known reference compounds. The general appearance of the mass spectra points to a homoconjugated arrangement of double bonds (Blumer et al. 1970; Youngblood et al. 1971; Karunen 1974; Steiger et al. 2007). The mass spectra of these compounds show the characteristic fragments a, a′, and b, which, together with the molecular mass, allow assignment of double bonds (Fig. 1). The spectra are shown in the Supplementary material (S1).

Chemoinformatics Characteristic cuticular hydrocarbons for "within-nest groups" of animals (workers, callows, larvae, pupae, spiders, or silverfish of a particular host colony) were identified and compared for potential similarities with multivariate statistical methods. To consider a GC peak characteristic for a particular within-nest group, at least 50% of the samples belonging to this group had to show the corresponding GC peak. Peaks had to be characteristic for at least one group; otherwise, they were considered not consistent and excluded from analysis. Consistency of 50% of the samples within one group is a rather conservative restriction and therefore minimizes the risk of excluding meaningful chemicals from analysis. Contaminants were excluded, and only hydrocarbons of 21 C-atoms or more were included in the statistical evaluation, since these are considered typical insect cuticular hydrocarbons (Carlson et al. 1998). Absolute peak area of these substances was fourth root transformed, and each peak analyzed in a sample was standardized by dividing its area by the transformed area of the maximum peak in the sample to evaluate relative proportions of compounds only. We used this procedure because we had no a priori hypothesis about the composition of profiles between host and symbionts, and standardization by maximum peak area appears most appropriate for comparing profiles containing different numbers of peaks. Non-parametric statistical procedures robust to data type and distribution (Clarke 1999) were applied by using the Primer 6 software package (Primer-E Ltd.). Bray–Curtis distances were calculated, which were then subjected to the following statistical procedures. A similarity percentage (SIMPER) procedure was used to calculate relative contributions of each compound to the similarity of a within-nest group. Non-metric multidimensional scaling (NMDS) was applied to visualize chemical distances. These were analyzed further by hierarchical cluster analysis (CA), with group average cluster mode, and the results were combined with the NMDS plots to visualize similar groups of samples. A priori hypotheses about differences between sample groups were tested for significance by the analysis of similarities (ANOSIM).

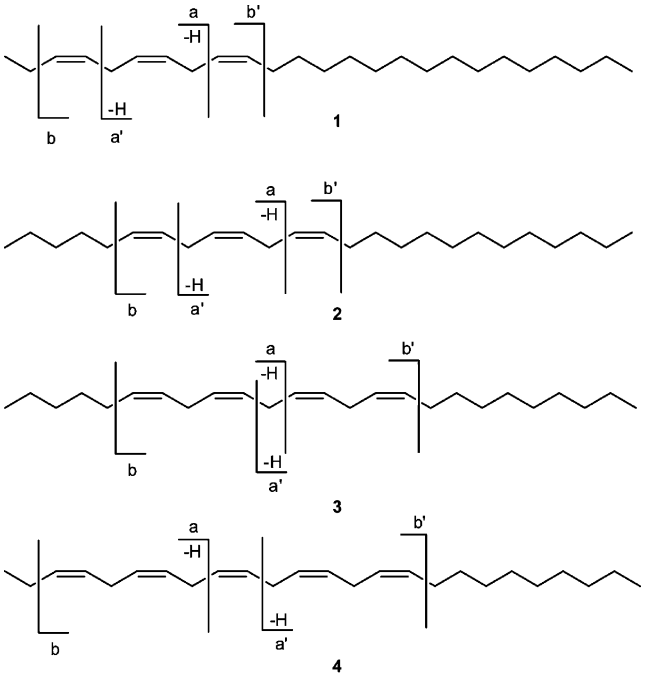

Fig. 1 Characteristic fragments for the identification of homoconjugated polyenes. Ions of the general formula $[C_nH_{2n-4}]^+$.are preferentially formed (*a*, *a′*), resulting in intense even-numbered ions. Less abundant were ions resulting from other characteristic cleavages next to the polyene system, such as cleaving off the shorter alkyl chain (*b*) or cleavage in the distal end of the polyene system resulting in less abundant ions (*b′*), often not more intense than other ions of the same ion series. For example, by this method, 3,6,9-pentacosatriene (*1*, *a*=108, *a′*=290, *b*=317=M-29) can be readily differentiated from 6,9,12-pentacosatriene (*2*, *a*=150, *a′*=248, *b*=275=M-71). Similarly, 6,9,12,15-pentacosatetraene (*3*, *a*=150, *a′*=206, *b*=273=M-71) can be differentiated from the 3,6,9,12-isomer (*a*=108, *a′*=248, *b*=315=M-29). Finally, the spectrum of 3,6,9,12,15-pentacosapentaene (*4*, *a*=108, *a′*=206, *b*=313=M-29) was identical to the published spectrum of the all-*Z* isomer (Leal et al. 2005), also showing an identical retention index. With the exception of 1, all tri-, tetra-, or pentenes were present in trace amounts only (Table 2). Not all trace components could be detected in samples from spiders and silverfish because they usually contained much less material, making identification of trace compounds difficult

Results

We collected myrmecophiles from ten different *L. distinguenda* colonies and recorded a median of three spiders

(range, 1–13) and 14 silverfish (range, 3–47). This difference in abundance is significant (P=0.008, exact Wilcoxon matched-pair test, N=10 each). It is important to note that emigrations could not always be sampled from the very beginning so that higher total numbers of myrmecophiles are possible and even likely. Nevertheless, silverfish are found typically in the earlier emigration phases and spiders in later phases, so that the number of silverfish was likely underestimated relative to the number of spiders. Consequently, sampling error should not affect the observed difference in abundances. On three emigrations, larval packets carried by worker ants were collected, which contained silverfish larvae (size, 1.0–1.2 mm) hidden between host larvae, as well as silverfish subadults (size, <4 mm) attached to the outside of the packets. This suggests that the entire life cycle of this myrmecophile takes place within the host colonies.

Behavioral Integration Surprisingly, we observed considerable differences in survival rates of the myrmecophiles in our laboratory colonies, which contradict the naturally observed abundances. Within the first 6 days of observation, 92–100% of the spiders survived (N=20), which was similar to host worker survival of 80–100%. In contrast, silverfish (N=41) disappeared almost completely within these 6 days (0–30% survival), and this loss was attributed to increased host aggression. In all laboratory nests, aggression increased toward silverfish, and active killings of myrmecophiles were frequently observed (Fig. 2). Thus, the observed mortality of silverfish was not a result of inappropriate laboratory handling, but rather a consequence of host aggression (see below). We observed significantly more attacks toward silverfish than spiders (P<0.001, U= 5211.5, Mann–Whitney U test, N=54).

Overall, the two myrmecophile species showed strongly dissimilar behavioral patterns (P<0.001, X^2 test, Table 1). Spiders interacted more with adult ants (P<0.001, Mann-Whitney U test, U=3132.0, N_{SP}=271, N_{SF}=147), whereas silverfish interacted more frequently with callow adults (P= 0.001, U=2,265.5, Mann–Whitney U test, N_{SP}=174, N_{SF}=72). Silverfish were frequently observed moving their body surface directly over the cuticle of adult and callow worker ants. This type of close physical contact to the host may be a mechanism that obtains host cuticular chemicals (Lenoir et al. 1997, 2001). This characteristic behavior of silverfish was more frequent during interaction with callow adult workers than with adult ants (P<0.001, U=4,842.9, Mann–Whitney U test; N=31). In accordance with this observation, aggression toward myrmecophiles (N=87) was never initiated by callows but instead by older workers. Contact between silverfish and worker ants occurred most frequently when the silverfish were below the ants (Table 1) and when the ants were occupied with other tasks such as feeding or brood care. Spiders, in contrast, were typically observed crawling on top of adult workers or callows and actively maintained contact by following worker ants through the nest (Table 1). From the on-top position, spiders had permanent physical contact and frequently moved their legs actively over the cuticle of the host (N> 20). Both myrmecophiles spent significant time in areas where ants were actively feeding and also were observed directly in contact with food (Table 1). The rare occasions when myrmecophiles directly contacted food with their mouthparts (Fig. 3) lasted only a few seconds each.

Aggressive interactions were regularly observed between spiders; however, these interactions did not result in injury or fatality. On encounter, one spider typically displaced another individual after a short period of leg-struggling. Spiders occasionally constructed small, flat webs (0.5–1 cm diameter) inside the ant nests, but prey capture was never observed with these webs. Passing host ants also frequently damaged these webs; therefore, they were of short durability and might serve purposes other than foraging.

In exchange experiments (see "Methods and Materials"), *L. distinguenda* recognized conspecifics from other colonies as alien; however, they were treated with low aggression (Fig. 4a). In addition to inspection behavior, which included intense bouts of antennation (N=330), dominance behavior was displayed, which included upright positioning of the resident ant, mandible grabbing and

Fig. 2 Myrmecophiles are attacked and killed by host *Leptogenys distinguenda* ants. *Left* Myrmecophilic spider, *Gamasomorpha maschwitzi*; *right* myrmecophilic silverfish, *Malayatelura ponerophila*

Table 1 Relative proportions of typical behaviors observed for spiders, *Gamasomorpha maschwitzi*, and silverfish, *Malayatelura ponerophila*, in two *Leptogenys distinguenda* laboratory colonies

Behavior	Spider	Silverfish
At larvae	9.7	3.0
At pupae	10.1	0.7
On callows (contact)	11.8	35.0
On adults (contact)	9.2	2.8
Rubbing on callows	0.0	5.6
Rubbing on adults	0.0	1.0
Following adults	19.1	0.8
Below adults (contact)	0.9	12.8
Attacked by adults	1.7	2.5
Groomed by adults	1.2	0.0
At food	33.0	33.5
Feeding	3.2	2.5
Total	100.0	100.0

holding down of the subdominant, and antennal boxing (N=13). These interactions ceased within 5 min so that introduced workers could finally move undisturbed and were adopted into the alien colony. In contrast, allospecific ants (*Leptogenys* sp.) were treated aggressively (Kruskal–Wallis test, P<0.001, N=128). Resident *L. distinguenda* workers displayed defensive and aggressive behavior, including bouts of forward jerking (N=214) and biting (N=165). Allospecific workers were found dead, although this happened long after the 10-min observation period had ended. In contrast, and similar to conspecific workers, introduced myrmecophiles from other *L. distinguenda* colonies were treated with lower aggression (Fig. 4a). Both myrmecophilic species sought direct physical contact with resident ants, possibly thus obtaining chemical signatures (Fig. 4b, Kruskal–Wallis test, P<0.001, N=82). However, behavioral responses to host aggression differed considerably between the two myrmecophilic species, which explains observed differences in contact frequencies. Spiders remained stationary, tolerated ant dominance behavior, and tried to maintain host contact (N=58). Silverfish, in contrast, attempted to escape when encountered, a behavior that provoked aggressive chases and attacks by ant workers, similar to the response to introduced allospecifics (N=8). If resident ants were able to capture an alien silverfish, it was subsequently killed. Thus, the survival of transferred silverfish was zero (N=3), whereas eight of ten spiders were finally accepted.

Chemical Integration We identified 109 different cuticular hydrocarbons among the host and two myrmecophiles (Table 2). Many of the substances occurred in low amounts, so that only characteristic principal compounds were included in the multivariate analysis (see "Methods and Materials"). The analysis showed, however, that spiders and silverfish did not carry any compounds that were uniquely different from those on the host ants (Table 2). Furthermore, the presence or absence of cuticular hydrocarbons on the myrmecophiles depended on their concentrations on the host ants. Only compounds that occurred as traces on the host were found significantly less frequently on the myrmecophiles than expected under the hypothesis of a total match of compounds (P<0.001, chi-square goodness of fit test, N=75) (see Supplementary material, Table S2). All compounds that occurred at >0.1% of total peak area were not found less frequently than expected (P>0.545, chi-square goodness of fit test, N=119, 27, and 12, respectively).

The hydrocarbon profiles from workers of three different *L. distinguenda* laboratory colonies showed weak (Fig. 5), but significant, colony-specific structure (ANOSIM, pairwise R values ranged between 0.23 and 0.67, overall P< 0.002). Hydrocarbon differences were more pronounced between colonies of *L. distinguenda* and *Leptogenys* sp. (ANOSIM, R>0.94, P<0.001; Fig. 5). Further quantification is shown in the Supplementary material (Table S3).

The hydrocarbon profiles of myrmecophilic spiders and silverfish from six field colonies resembled the patterns from their host to a high degree; however, colony-specific matching was not observed. This might be due in part to the small sample sizes of the myrmecophiles. More likely and

Fig. 3 Myrmecophile contact with food lasts only a few seconds. *Left* Myrmecophilic spider *Gamasomorpha maschwitzi* gets brief access to a prey item from the top. *Right* myrmecophilic silverfish *Malayatelura ponerophila* participates from the top at larval feeding

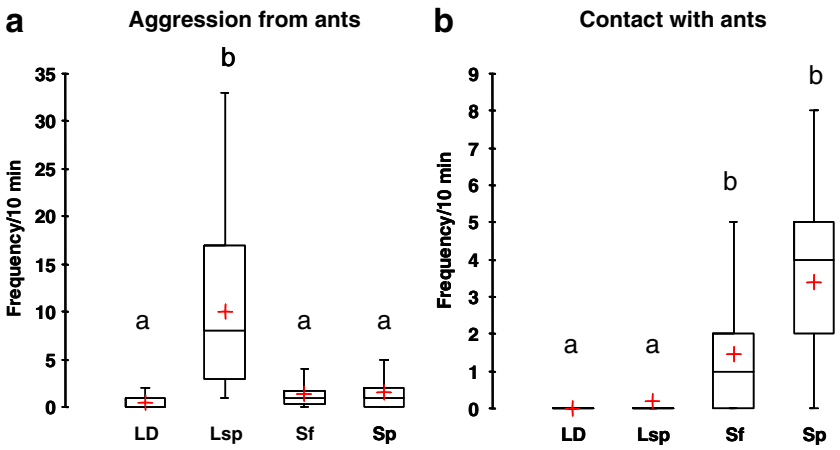

Fig. 4 Differences in aggressive behavior directed by and contact received by *Leptogenys distinguenda* workers during 10-min observation periods relative to conspecific workers (*LD*), allospecific workers (*Lsp*), and myrmecophilic silverfish (*Sf*) or spiders (*Sp*). **a** Aggression of resident ants toward introduced animals. **b** Physical contact initiated by introduced animals to resident ants. The *plus symbol* indicates mean values. *Different letters* denote significant differences according to a Kruskal–Wallis test, $P<0.001$

consistent with the results above, *L. distinguenda* intercolonial differences are too weak to be detected through their myrmecophilic fauna. Instead of showing colony-specific structure, the profiles of within-nest groups were distinct across all *L. distinguenda* colonies ($R=0.376$, $P<0.001$, two-way nested ANOSIM, within-nest groups nested in colony groups). Thus, we further compared chemical similarities among workers, callow adults, larvae, pupae, as well as myrmecophilic spiders and silverfish of field colonies and found a high chemical resemblance of the two myrmecophile profiles with callow and adult host profiles (Fig. 6). Nine cuticular hydrocarbons accounted for more than 98% of the similarity within each of these groups, despite being present in different proportions (see results of SIMPER analysis in the tabular insert of Fig. 6).

Both myrmecophiles were chemically more similar to adult and callow adult host ants than to host ant larvae and pupae, which typically lack most cuticular hydrocarbons (Fig. 7). Based on hierarchical CA, 98% of adults, 97% of callow adults, 84% of silverfish, and 57% of spiders were arranged in a 70% similarity group, whereas only 38% of the larvae and none of the pupae were sorted into the same group. Instead, all pupae, most larvae, and the remaining samples were spread in six separated groups. On a higher similarity level of 80%, there were still 88% of adults and callows, as well as 57% of silverfish, and 21% of spiders sorted into one group. A second group contained few host ants (6% of workers and 9% of callows) and a considerable number of spiders (36%) and silverfish (19%). An ANOSIM revealed more details, which cannot be observed directly in Fig. 7. Spiders were less similar to both callows ($R=0.54$) and adults ($R=0.58$), whereas silverfish showed higher resemblance to callows ($R=0.12$) and adult host ants ($R=0.22$) ($P<0.002$ for all pairs, ANOSIM). Thus, in the six field colonies, silverfish were chemically more similar to their hosts than were spiders, whether we performed the ANOSIM on selected cuticular hydrocarbon groups only (i.e., unsaturated or saturated) or on the most abundant hydrocarbons.

Discussion

Social Integration Strategies of Myrmecophiles If mimicry is regarded as the resemblance of the properties of a model organism (Dettner and Liepert 1994), our results demonstrate chemical integration by mimicry of host cuticular hydrocarbon patterns for both myrmecophilic species. To the best of our knowledge, this is the first report of chemical mimicry in silverfish. A well-known example of chemical mimicry in spiders is with *Cosmophasis bitaeniata*, which feeds on larvae of the weaver ant, *Oecophylla smaragdina*, and acquires a colony-specific chemical signature (Allan et al. 2002; Elgar and Allan 2004, 2006). Nonetheless, social integration of most myrmecophilous spiders remains poorly studied, even though myrmecophily is not uncommon among spiders (Cushing 1997). Our study supports the notion that a passive mechanism of chemical mimicry through maintenance of physical contact (Lenoir et al. 1997, 2001) was used by both myrmecophiles. Specifically, a passive transfer of chemicals predicts a match of major host compounds with only host compounds of low concentrations falling below the detection threshold on the myrmecophiles. Furthermore, it appears evolutionarily unlikely that spiders and silverfish experienced all mutations to biosynthesize all or most of these specific *Leptogenys* host compounds (Dettner and Liepert 1994). A system of passive chemical mimicry by adoption of nest odor requires a constant renewal of the myrmecophile cuticular hydrocarbon profile, and such efforts were apparent in their behavior. In both species, over 40% of all monitored behaviors were species-specific attempts to get in close contact with host ants. Direct physical contact with legs (spiders) or body surface (silverfish) was observed frequently. Nevertheless, our chemical analysis of free-living colonies demonstrated considerable variation in the chemical profiles within each group of myrmecophiles, suggesting different success in maintaining mimicry among individuals. Chemical dissimilarity even reached

Table 2 Hydrocarbons identified in workers of the host ant *Leptogenys distinguenda* and the myrmecophilic spider, *Gamasomorpha maschwitzi*, and silverfish, *Malayatelura ponerophila*

Compound	RI	Host[a]	Spider[a]	Silverfish[a]
Octadecane	1,800	tr	nd	nd
Eicosane	2,000	tr	nd	nd
Heneicosane	2,100	x	x	x
Docosane	2,200	x	x	x
2-Methyldocosane	2,261	tr	nd	nd
Tricosane	***2,300***	***xxx***	***xxx***	***xxx***
Tricosenes		*11*; **Z9**; *E9*; **Z7**; *E7*; *6*; 5; *3*; 1	9; 7	9; **7**; 5
	2,273	x	x	x
	2,280	***xx***	***xx***	***xx***
	2,290	x	x	x
6,9-Tricosadiene	2,269	tr	nd	nd
3,6,9-Tricosatriene	2,277	tr	tr	nd
2-Methyltricosane	2,361	x	x	x
5-Methyltricosane	2,350	tr	nd	tr
7-Methyltricosane	2,340	tr	nd	nd
9-Methyltricosane	2,336	x	tr	tr
Tetracosane	***2,400***	***x***	***x***	***xx***
Tetracosene		5-11; 1	6	9; 8
	2,379	x	tr	x
	2,387	tr	tr	tr
	2,393	tr	tr	tr
2-Methyltetracosane	2,463	x	x	x
9-Methyltetracosane	2,438	x	x	x
Pentacosane	***2,500***	***xx***	***xx***	***xx***
Pentacosene		**9**; **7**; *6*; 5; *3*; 1	**9**; **7**; 5; 1	**9**; **7**; 5;*1*
	2,477	***xx***	***xx***	***xx***
	2,485	***xxx***	***xxx***	***xxx***
	2,494	***xx***	***xx***	***xx***
6,9-Pentacosadiene	***2,473***	***xx***	***x***	***xx***
3,6,9-Pentacosatriene	2,481	x	x	x
6,9,12-Pentacosatriene	2,464	tr	nd	nd
3,6,9,12-Pentacosatetraene	2,434	tr	tr	tr
6,9,12,15-Pentacosatetraene	2,460	tr	nd	nd
3,6,9,12,15-Pentacosapentaene	2,442	x	tr	tr
2-Methylpentacosane	2,562	x	x	x
5-Methylpentacosane	2,550	x	x	x
7-Methylpentacosane	2,541	tr	tr	tr
9-Methylpentacosane	2,536	x	x	x
11-Methylpentacosane	***2,534***	***x***	***x***	***x***
Hexacosane	2,600	tr	tr	tr
Hexacosene		10; **9**; **8**; 7; 6	nd	8
	2,579	x	tr	x
	2,588	x	nd	x
Hexacosadiene	2,573	tr	tr	nd
Heptacosane	2,700	x	x	x
Heptacosene		**9**; 7; *5*; *1*	**9**; 7; *1*	**9**; 7
	2,678	tr	tr	tr
	2,684	***xxx***	***xxx***	***xxx***

Table 2 (continued)

Compound	RI	Host[a]	Spider[a]	Silverfish[a]
	2,694	***x***	***tr***	***tr***
6,9-Heptacosadiene	2,674	x	x	tr
3,6,9-Heptacosatriene	2,683	xx	x	x
6,9,12-Heptacosatriene	2,653	x	tr	tr
6,9,12,15-Heptacosatetraene	2,632	x	tr	tr
3,6,9,12,15-Heptacosapentaene	2,640	tr	tr	tr
2-Methylheptacosane	2,765	x	x	x
3-Methylheptacosane	2,777	x	x	tr
7-Methylheptacosane	2,742	x	x	x
9-Methylheptacosane	2,739	x	x	x
11-Methylheptacosane	2,733	x	x	x
7,13-Dimethylheptacosane	2,775	tr	tr	tr
Octacosene		10; **9**; 8; 7; 5	9; *8*	*9*
	2,780	x	nd	nd
	2,784	x	x	tr
	2,794	x	nd	tr
Nonacosene		**9**; 7 ??	9; 7	9
	2,880	tr	tr	nd
	2,888	***xxx***	***xx***	***xx***
	2,894	***xx***	***x***	***nd***
6,9-Nonacosadiene	2,881	tr	tr	tr
3,6,9-Nonacosatriene	2,889	x	tr	tr
6,9,12-Nonacosatriene	2,860	xx	tr	nd
6,9,12,15-Nonacosatetraene	2,837	x	tr	tr
3,6,9,12,15-Nonacosapentaene	2,846	tr	tr	tr
11-Methylnonacosane	2,935	x	x	tr
13-Methylnonacosane	2,934	x	x	tr
15-Methylnonacosane	2,934	x	tr	tr
7,13-Dimethylnonacosane	2,972	x	tr	nd
11,15-Dimethylnonacosane	2,960	x	tr	nd
Triacontene	2,988	x	tr	tr
		9; *7*	9	9
Hentriacontene	3,074	x	x	nd
6,9-Hentriacontadiene	3,085	xx	nd	nd
Hentriacontadiene	3,085	xx	nd	nd
3,6,9-Hentriacontatriene	3,093	tr	tr	nd
6,9,12-Hentriacontatriene	3,062	tr	tr	nd
6,9,12,15-Hentriacontatetraene	3,039	x	tr	nd
3,6,9,12,15-Hentriacontapentaene	3,048	x	nd	nd
13-Methylhentriacontane	3,127	tr	tr	nd
15-Methylhentriacontane	3,127	tr	tr	nd
7,15-Dimethylhentriacontane	3,170	tr	nd	nd
13,17-Dimethylhentriacontane	3,152	tr	nd	nd
9-Tritriacontene	3,283	x	tr	tr
6,9-Tritriacontadiene	3,280	tr	nd	nd
6,9,12-Tritriacontatriene	3,260	tr	nd	nd
6,9,12,15-Tritriacontatetraene	3,233	tr	nd	nd
13-Methyltritriacontane	3,327	tr	tr	nd
15-Methyltritriacontane	3,327	tr	tr	nd

Table 2 (continued)

Compound	RI	Host[a]	Spider[a]	Silverfish[a]
17-Methyltritriacontane	3,327	tr	tr	nd
7,15-Dimethyltritriacontane	3,368	tr	nd	nd

RI retention index

[a] Relative abundances of individual components within samples are denoted by the following abbreviations: *xxx* >10%, *xx* 1–10%, *x* 0.1–1%, and *tr* <0.1% of the total peak area. Location of double bonds of monoenes obtained by analysis of DMDS-derivatized samples are indicated in the appropriate entries. Usually three alkene peaks are observed, but peak assignments could not be made. In the less concentrated samples of spider and silverfish, the location of the double bond in the alkenes was only possible for major components. Typeface refers to the relative proportion within an entry (*bold* major isomer, *italics* minor isomer). *Numbers* indicate double bond positions. *Un* unknown position of double bonds, *nd* not detected. Compounds used in multivariate statistical analyses are rendered in bold italics.

such a high degree that several individuals were not grouped together with their host ants by CA. Such significant deviations from the host colony odor could provide sufficient recognition cues for the myrmecophile to be detected and rejected, which also explains the observed killings of myrmecophiles in our laboratory colonies.

In native colonies, silverfish resembled host profiles better than spiders, no matter which class of cuticular hydrocarbons the analysis was based on. This finding suggests that silverfish should thrive well in free-living host colonies, and in fact, they were found in high numbers. In stark contrast to this, silverfish showed strikingly high mortality rates in our laboratory colonies. This could be attributed only to the altered nest structure of our observation nests. Full-sized *L. distinguenda* colonies show a different and distinct nest structure (Witte 2001). Adult workers occupy peripheral areas of the nest, whereas large piles of pupae and dense aggregations of callow adults with larvae in their mandibles are found in the center. Silverfish prefer these central regions, where they find enough space to hide between brood, and to update their chemical profiles from defenseless callows without disturbance. Furthermore, they are able to hide their offspring in the larval clumps that are located in these central nest regions. Brood and callows were not arranged in such distinct spatial structure in our small laboratory colonies, so that silverfish were probably unable to find their natural niche. Encounters with aggressive mature workers were frequent, which constantly disturbed the silverfish and probably hindered the updating of their cuticular hydrocarbon profiles by physical contact. Consequently, they were detected, attacked, and finally killed. Mortality due to worker aggression was also observed in myrmecophilous silverfish associated with ecitonine army ants (Rettenmeyer 1963). In contrast to silverfish, most of the spiders were accepted in our *L. distinguenda* laboratory colonies probably due to their behaviorally different integration strategy, which depended less on the nest structure and interactions with defenseless callows. Continuous contacts with callow and adult host ants and frequent rubbing of their filigree legs over the host cuticle while resting directly on their bodies facilitated a constant update of the chemical signature. Spider cuticular profiles were presumably more stable under laboratory conditions, and these myrmecophiles were better suited to remain socially accepted.

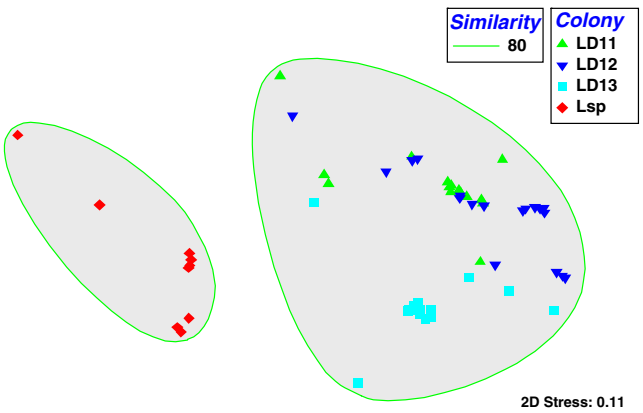

Fig. 5 Non-metric multidimensional scaling (NMDS) plot visualizing chemical similarities of three *Leptogenys distinguenda* colonies (*LD11*, *LD12*, and *LD13*) and one closely related *Leptogenys* sp. colony (*Lsp*) as an outgroup. Hierarchical cluster analysis also demonstrated the similarity among groups at the 80% level. N_{LD11}=15, N_{LD12}=18, N_{LD13}=17, and N_{Lsp}=9

Countermeasures by the Host Even if amplified by our laboratory conditions due to an artificial change in nest structure, the possibility of recognition and aggressive rejection of myrmecophiles is an important finding. This demonstrates that the host ant *L. distinguenda* is able to detect and regulate its symbiont fauna to a certain extent. Myrmecophiles live under the potential risk of being detected, expelled, or even killed. Accordingly, active host regulation can explain, on the one hand, the overall limited numbers of myrmecophiles within *L. distinguenda* colonies and, on the other hand, the large variation in myrmecophile abundances. Due to colony-specific circumstances and species-specific integration mechanisms, it might be temporarily more or less challenging for myrmecophiles to exploit a host colony. Although field conditions were

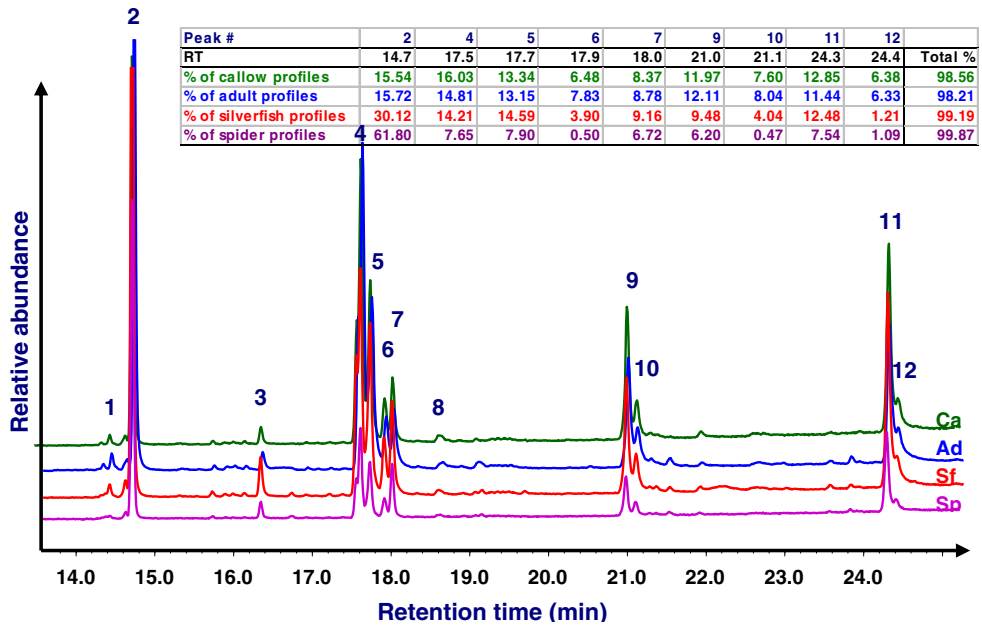

Fig. 6 Similarity of cuticular hydrocarbon profiles from the ant, *Leptogenys distinguenda*, the silverfish, *Malayatelura ponerophila*, and the spider, *Gamasomorpha maschwitzi*. Total ion chromatograms of (from *top* to *bottom*) callow (*Ca*), adult worker (*Ad*), myrmecophilic silverfish (*Sf*), and myrmecophilic spider (*Sp*). Principal compounds are numbered: *1* 9-tricosene, *2* tricosane, *3* tetracosane, *4* 6,9-pentacosadiene, *5* 9-pentacosene, *6* 7-pentacosene and 3,6,9-pentacosatriene, *7* pentacosane, *8* 9- and 11-methylpentacosane, *9* 9-heptacosene and 6,9-heptacosadiene, *10* 7-heptacosene and 3,6,9-heptacosatriene; *11* 9-nonacosene and 6,9-nonacosadiene; *12* 7-nonacosene and 3,6,9-nonacosatriene. The *table insert* shows relative contributions of the nine principal compounds to the similarity of within-nest group chemical profiles according to a similarity percentage (SIMPER) procedure (see text for details)

different from laboratory conditions, nest demography also varies in free-living *L. distinguenda* colonies. We suggest that silverfish can also experience integration difficulties under natural conditions (e.g., after demographic changes due to resource shortages, in small colony propagules after nest fission, or simply if a silverfish fails to locate callow adult aggregations in a larger colony or a widely spread nest arrangement). The considerable variation of silverfish abundance (three to 47 individuals) is in accord with this assumption. Although exploitation of colony resources by symbionts can be tolerated when they occur in low numbers, cost of symbionts to the colony increases if they occur in higher abundances. Consequently, there should be selection on the host for symbiont recognition and rejection mechanisms. The question remains to be explained why silverfish occur regularly in higher numbers than spiders in natural *L distinguenda* colonies. We hypothesize that the reproductive rate of silverfish mainly is responsible, because, in contrast to spiders, we repeatedly detected juvenile silverfish. Furthermore, spiders might encounter stronger intraspecific competition as they frequently showed intraspecific aggressive interactions.

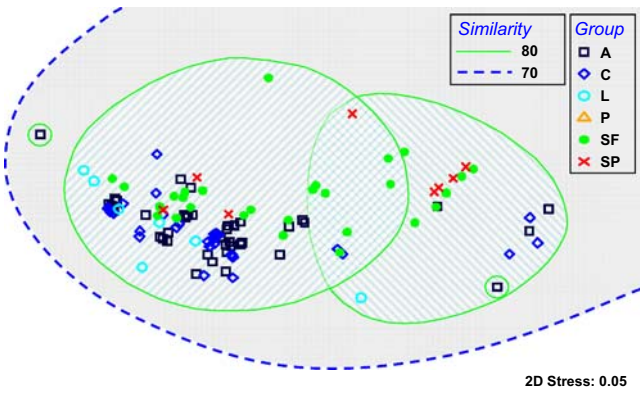

Fig. 7 Non-metric multidimensional scaling (NMDS) plot visualizing chemical similarity of 49 adults (*A*), 32 callow adults (*C*), 21 larvae (*L*), 11 pupae (*P*), 37 myrmecophilic silverfish (*SF*), and 14 myrmecophilic spiders (*SP*) from six different *Leptogenys distinguenda* colonies (=164 samples). In the hierarchical cluster analysis, the 70% similarity group contained 77% of all samples (=126 samples). Pupae (*P*) data points are out of range of the shown frame

Nestmate Recognition and Aggression Somewhat surprisingly and atypically for ants, nestmate recognition appeared to be developed weakly in *L. distinguenda,* which contradicts an effective symbiont recognition system. Both variation in cuticular hydrocarbon profiles between different colonies and the level of inter-colonial aggression were surprisingly low in *L. distinguenda*. This phenomenon might be explained by the mode of reproduction in this species. Winged males disperse to enter alien colonies for mating, whereas queens are wingless. Similar to our

exchange experiments with conspecifics, male intrusion into alien colonies elicits initial aggression that ceases over time so that males are finally able to enter (Witte, personal observation). Furthermore, males show similar profiles to workers (Witte, unpublished data), and this suggests a trade off in chemical discrimination and aggressive behavior in *L. distinguenda*. Cuticular hydrocarbon profiles might be just sufficiently different to recognize aliens and to maintain colony integrity but not so different to elicit high aggression levels, which in turn would complicate the intrusion and acceptance of males. Nevertheless, social integrity is guaranteed, as our analysis confirmed that chemical differences were greater between different *Leptogenys* species, accompanied by elevated aggression. In addition to chemical recognition and rejection, there are other lines of defense against social parasites, such as the extraordinarily high migration frequency of *L. distinguenda* (Maschwitz et al. 1989) that places additional adaptive challenges on the symbiont fauna. However, both spiders and silverfish appear well-adapted in this respect, as they are able to follow host emigration trails independently and without obvious difficulties (Witte et al. 2008). Thus, the mechanisms based on chemical recognition and rejection that we described in this paper appear to be of particular importance concerning the regulation of these myrmecophile species.

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Nestmate and Task Cues are Influenced and Encoded Differently within Ant Cuticular Hydrocarbon Profiles

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Abstract Insect cuticular hydrocarbons (CHCs) are primarily antidesiccation agents, but they also play crucial roles in intra- and interspecific communication, especially among social Hymenoptera. The complex CHC profiles of social insects have often been compared among individuals, kin, nestmates, colonies, and species. In the ant *Formica exsecta*, only the (*Z*)-9-alkene part of the CHC profile encodes the nestmate signal. Here, we showed that the other major part of the CHC profile with *n*-alkane components is influenced strongly by the task a worker performs (foraging vs nonforaging). This part of the profile is independent of the nestmate signal. Therefore, the CHC profile of *F. exsecta* workers is composed of two independent parts: a colony-specific (*Z*)-9-alkene profile under genetic influence and an environmentally influenced task-related *n*-alkane profile. The dissociating of the CHC profile into two or more independent parts has implications for the analysis and interpretation of past and future CHC studies.

Keywords Alkanes · Task recognition · *Formica* · Ants

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Introduction

The cuticle of all life stages of insects is covered with a thin epicuticular layer of wax. In the vast majority of species, this layer is dominated by hydrocarbons, which are environmentally stable (Lockey 1988; Gibbs and Crockett 1998; Martin et al. 2009). Hundreds of different insect cuticular hydrocarbons (CHCs) have now been described, and a major challenge in chemical ecology is to understand their various roles. The principal role of CHCs is the control of the transcuticular water flux, thereby preventing wetting, dehydration, and the penetration of microorganisms. CHCs account for about 0.1% of the total mass of a typical insect, but their presence can reduce the insect's permeability to water by up to 1,300% (Edney 1977). Structurally, the best hydrocarbons to perform this task are saturated straight-chained linear *n*-alkanes that dominate the CHC profile of nearly all insects (Blomquist and Dillwith 1985; Lockey 1988). However, many other more complex CHCs are present and, together with *n*-alkanes, are believed to play important roles as semiochemicals in insect communication (Howard and Blomquist 2005).

The complexity of CHC profiles has led to the widespread use of multivariate statistical methods, techniques that reduce data dimensionality and so confound rather than separate out the various signals (Martin and Drijfhout 2009). By using these methods, researchers have suggested that the variation in CHC profiles is associated with recognition cues at the individual (e.g., D'Ettorre and Heinze 2005), kin (e.g., Arnold et al. 1996), patriline (Boomsma et al. 2003), nestmate (e.g., Nielsen et al. 1999; Wagner et al. 2000; Liu et al. 2001; Kaib et al. 2002), and species level (e.g., Lockey and Metcalfe 1988; Neems and Butlin 1995; Dapporto 2007). Furthermore, correlations have been found between changes in the CHC profiles and

caste (e.g., Ayasse et al. 1999; Tentschert et al. 2002), dominance (e.g., Ayasse et al. 1995; Heinze et al. 2002; Monnin et al. 2002), task (Greene and Gordon 2003), and fertility (e.g., Liebig et al. 2000). All these studies have assumed that the profile perceived by the insect and that produced by the gas chromatography (GC)–mass spectrometry (MS) are congruent. However, it is becoming apparent that this is not the case. For example, honeybees learn and discriminate some hydrocarbons well (alkenes) and others poorly (*n*-alkanes) (Châline et al. 2005). This explains why honeybees were attacked when their alkene profile was changed but were ignored when their *n*-alkane profile was altered (Dani et al. 2005), even though the honeybee profile was dominated by *n*-alkanes (Arnold et al. 1996; Dani et al. 2005).

To make progress in recognition studies, it is essential that we detect those components of the CHC profile relevant for communication. In the seed-eating desert harvester ant (*Pogonomyrmex barbatus*), the cuticles of foragers and patrollers have higher amounts of *n*-alkanes than the cuticles of nonforagers (Wagner et al. 1998), and the proportion of *n*-alkanes increased when individuals were exposed to high temperatures and low relative humidity (Wagner et al. 2001). This indicates that *n*-alkane production is influenced by environmental conditions.

The ant *Formica exsecta* is an ideal model species because it has a simple CHC profile dominated (>90%) by an *n*-alkane and a (*Z*)-9-alkene homologous series (Martin et al. 2008a). We have shown previously that the (*Z*)-9-alkenes encode nestmate recognition in this species, whereas the proportions of *n*-alkanes among nestmates could be highly variable (Martin et al. 2008a, b). This phenomenon also occurs in other *Formica* species (*Formica lemani*, *Formica candida*) that have CHC profiles similar to *F. exsecta* (author's unpublished data). We investigated in five species of Formicinae ants whether the variability in *n*-alkanes is due to foragers that possess higher amounts of *n*-alkanes than nonforagers. We also studied in *F. exsecta* whether the *n*-alkane and colony-specific (*Z*)-9-alkene parts of the profile are independent.

Methods and Materials

Study Species During May 2008, we collected 10 foragers and 10 nonforagers from nine different *F. exsecta* colonies within 30 km of the Tvärminne zoological station at Hanko, southern Finland. Foragers were collected individually at a distance between 1 and 2 m from the nest mound. Care was taken not to disturb the mound until the foragers had been collected. Nonforagers were then collected by quickly removing nesting material from as deep within the mound as possible. Nest material was placed into a container and 10 ants were collected. It is impossible to guarantee that only nonforagers were collected, but collecting ants from deep inside the mound decreases the chance of collecting foragers. The presence of a small proportion of foragers in the nonforaging group will lead to an underestimation of the actual differences.

To investigate the generality of forager vs nonforager differences, we extended the study to include four other ant species: *Formica pratensis*, Finland; *Formica lugubris*, UK; *F. lemani*, UK; and *Lasius niger*, UK. All UK samples were collected in the Peak District (Derbyshire) during June 2008 by using the same collection method, i.e., collecting 10 foragers and 10 nonforagers, but only one colony per species was studied as we wished to establish whether a trend similar to that found in *F. exsecta* exists, rather than to conduct a full-scale study.

Chemical Analysis All individuals were killed after collection by storing in a freezer at −20°C. Each ant was placed into a glass insert with 30 μl (50 μl for *F. pratensis* and *F. lugubris*) of high-performance liquid chromatography-grade hexane. After 30 min, the hexane had evaporated. The ant was removed, and the dried extract was stored at 5°C. Immediately prior to analysis, 30 μl of hexane containing an internal standard (5 ng/μl of C_{20}) was added to the extract. The sample was analyzed on an HP 6890 GC (equipped with an HP-5MS column; length: 30 m; ID: 0.25 mm; film thickness: 0.25 μm) connected to an HP5973 MSD (quadrupole mass spectrometer with 70-eV electron impact ionization). Samples were injected in the splitless mode, and the oven was programmed from 70°C to 200°C at 40°C/min, and then from 200°C to 320°C at 25°C/min, and held for 2 min at 320°C. Helium was used as carrier gas at a constant flow rate of 1.0 ml min^{-1}. Wood ants of the *Formica rufa* group have CHC profiles that contain compounds not detected by standard columns (Akino 2006). Therefore, we ran the *F. pratensis* and *F. lugubris* samples on a SGE Forte HT5 GC capillary column (length: 30 m, inner diameter: 0.22 mm, and 0.1 μm film thickness) with the oven programmed to start at 50°C (1-min hold) with a first ramp of 40°C/min up to 280°C, then a 20°C/min ramp to 420°C with a 2-min hold. All compounds were characterized by using standard MS databases, diagnostic ions, and Kovats indices. Authentic standards of alkanes and alkenes were either purchased or synthesized at Keele University. The double-bond positions of the alkenes have been previously determined by dimethyl disulfide derivatization (Martin et al. 2008b).

Head Width Measurements Since large ants will have more CHCs relative to small ants, we investigated whether morphological size differences occurred among colonies and between foragers and nonforagers. These data enabled us to relate CHC quantities to body size. We compared the

head width of the 10 workers from each group (foragers vs nonforagers) in each colony. After the CHC had been extracted, each ant's head was removed and placed onto a microscope slide. Head width was measured with a Leica light binocular microscope at a magnification of 80× with a graticule.

Statistical Analysis The amounts of *n*-alkanes and alkenes extracted from each ant were determined by using the peak area of an internal standard (C_{20}) as the reference. For statistical analysis, the data were log-transformed in order to meet the assumption of equal variances (homogeneity of variance) before conducting two-way independent analyses of variance for the *F. exsecta* ants (factor 1: *n*-alkanes/alkenes; factor 2: colonies). Then, each *F. exsecta* colony was analyzed separately by using an independent *t* test to compare the chemical differences between quantities of *n*-alkanes and alkenes detected in extracts of foragers and nonforagers. The *t* test was conducted because the amount of CHCs extracted was highly correlated to head width (see results), and head width between *F. exsecta* colonies was highly variable (Fig. 1). Furthermore, across all individuals,

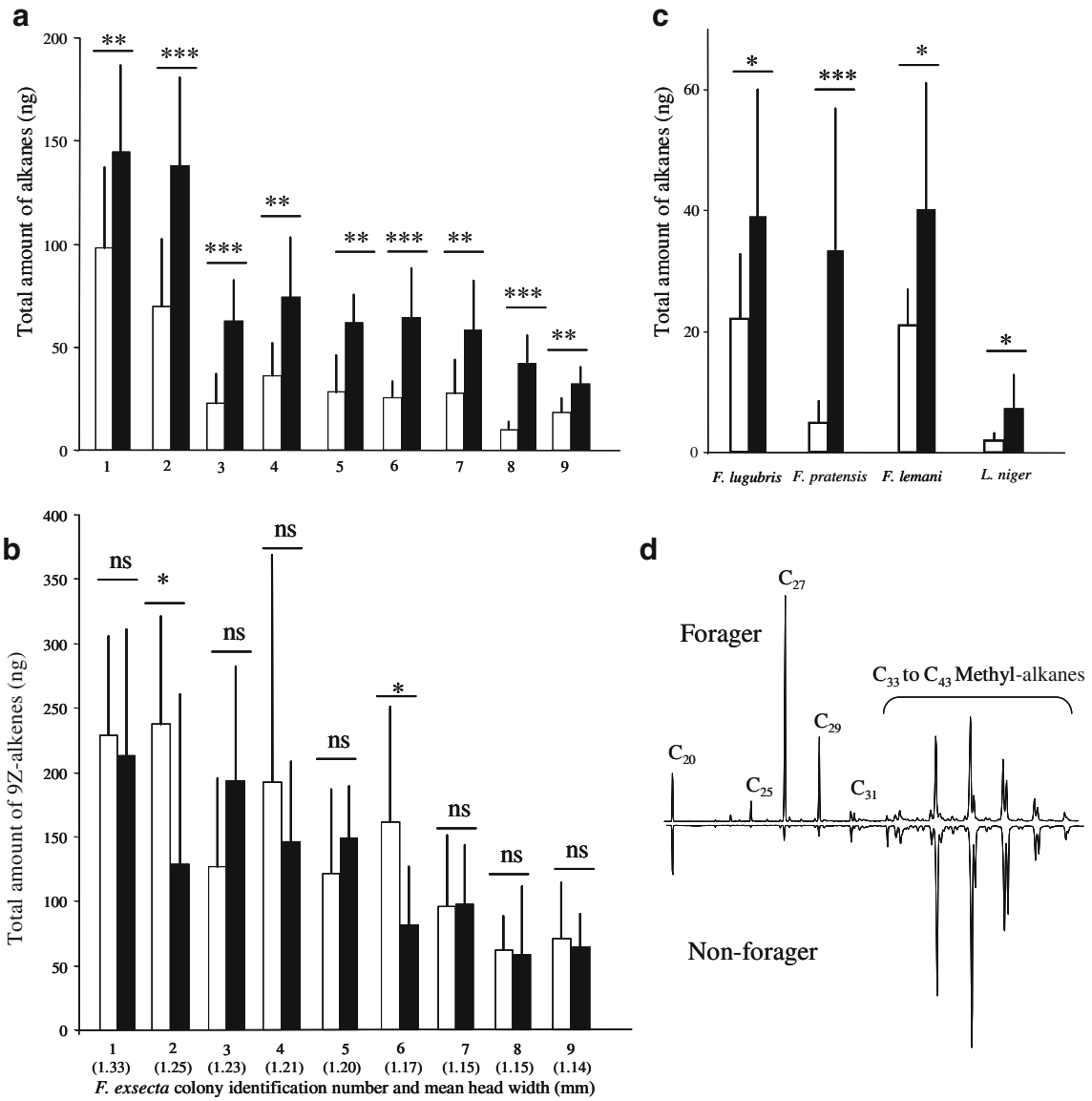

Fig. 1 Comparison of amounts (ng; means + SD) of **a** *n*-alkanes, **b** alkenes between nonforagers (*clear bars*, $N=10$) and foragers (*filled bars*, $N=10$) of nine different *F. exsecta* colonies. The numbering of colonies is ordered by decreasing worker head widths, which are given in parentheses. **c** The amounts of *n*-alkanes (ng; means + SD) in nonforagers (*clear bars*, $N=10$) and foragers (*filled bars*, $N=10$) in three additional *Formica* species and *L. niger*. **d** Part of typical total ion current chromatograms with *n*-alkanes (C_{25}–C_{31}) and C_{33}–C_{43} methyl alkanes from cuticular extracts of a *F. pratensis* forager and nonforager; C_{20} is the internal standard. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ was determined by independent *t* tests

the amounts (ng) of *n*-alkanes and alkenes in *F. exsecta* were poorly correlated (R^2=0.18, N=180). That indicates that the production of one is largely independent of the other. The correlations between amounts of CHCs (ng) and mean head width (mm) among the nine colonies were calculated in SPSS v. 14 by using Pearson's correlation coefficient. As each of the four other ant species studied were represented by a single colony, these were also analyzed individually with an independent *t* test to compare quantities of CHC in foragers and nonforagers. As in *F. exsecta*, the amounts of *n*-alkanes were poorly correlated to nonalkane CHC in the profiles of the four additional species (*F. lugubris* R^2=0.12, *F. pratensis* R^2=0.01, *F. lemani* R^2=0.19, *L. niger* R^2=0.22).

To illustrate how calculating CHC proportions influences the way we perceive CHC data, we first calculated the proportions in the usual way, i.e., the proportion of each individual CHC is based on the total amount of *all* CHCs in the profile. Then, we calculated the proportion in a new, more biologically realistic way since it separates out rather than combines potential cues: that is, the proportion of each individual CHC is calculated by using the total amount of CHCs belonging only to the *same homologous series*. The following formula provides an example for this method of evaluation:

$$C_{25}=\frac{C_{25}}{\sum n-\text{alkanes}}\ \text{rather than}$$

$$C_{25}=\frac{C_{25}}{\sum n-\text{alkanes}+\sum \text{alkanes}+\sum \text{methyls}\ldots}$$

Results

In each of the nine *F. exsecta* colonies, the amount of *n*-alkanes extracted from foraging ants was consistently greater than that from nonforaging ants (Fig. 1a). This difference was highly significant ($F_{1,157}$=164.8, P<0.001). However, there was no corresponding difference ($F_{1,\ 157}$= 2.8, P=0.095) in the amount of alkenes on foragers and nonforagers (Fig. 1b). The amounts of both *n*-alkanes and alkenes differed significantly between colonies (*n*-alkanes $F_{8,157}$=2203, P<0.001; alkenes $F_{\ 8,157}$=13, P<0.001) and were highly correlated with head width (see below). Across the nine *F. exsecta* colonies, foragers (N=90) had, on average, 2.3 times the amount of *n*-alkanes of that found on nonforagers (N=90).

A significantly (t always >2.2, df=18, P always <0.04) higher amount of *n*-alkanes on foraging vs nonforaging ants also was found in the additional four species investigated (Fig. 1c), indicating that this is a widespread phenomenon, at least among the Formicinae ants. In *F. pratensis*, the putative nestmate/species recognition compounds, i.e., methylated alkanes (Akino 2006), were well separated from the lighter *n*-alkanes that were not present at chain lengths greater than C_{33} (Fig. 1d).

Worker size among the nine *F. exsecta* colonies differed significantly ($F_{1,160}$=11.4, P<0.001), although the within-colony variation in head width was similar in each colony, i.e., the *SD* ranged from 0.04 to 0.09 with a mean of 0.07 mm. The amount of CHC (ng) extracted and worker head width correlated significantly across the nine colonies [Pearson's correlation, R=0.94, P (one-tailed)<0.001, N=9]. This explains the large intercolony variation in the amounts of *n*-alkanes (Fig. 1a) and alkenes (Fig. 1b). Head widths did not differ significantly ($F_{1,160}$=1.3, P=0.26) between foragers (mean=1.22±0.09 mm, N=90) and nonforagers (mean=1.21±0.10 mm, N=90) across all colonies, or when each colony was analyzed separately (t tests, P always >0.05).

Discussion

Here, we demonstrated that the *n*-alkane, but not the (*Z*)-9-alkene, components of the CHC profile of *F. exsecta* workers varied depending on task, i.e., foraging vs nonforaging. Across all *F. exsecta* colonies, and in single colonies of *F. lemani*, *F. lugubris*, *F. pratensis*, and *L. niger*, the amounts of *n*-alkanes on the cuticles of foragers were consistently greater than on the cuticles of individuals taken from inside the colony (Fig. 1a). Nevertheless, some individual exceptions from this rule were detected; the nonforaging groups occasionally contained some individuals with forager-typical *n*-alkane amounts. These individuals were not removed from the analysis of the nonforaging groups.

Alkanes are structurally the best hydrocarbons to function as desiccation resistance and waterproofing compounds (Blomquist and Dillwith 1985). Foragers, which are exposed to greater fluctuations in temperature and humidity, have increased amounts of protective *n*-alkanes, as has been shown in the ant *P. barbatus* (Wagner et al. 1998, 2001). These studies indicate that changes in the amount of *n*-alkanes were a direct response to environmental conditions, rather than age-related. In *F. exsecta*, workers emerge during a 1-month period in summer and survive for about a year (as determined by mark-recapture records, Emma Vitikainen, personal communication). Therefore, irrespective of task, all workers in a nest are of similar age. Furthermore, no size polymorphism related to foraging was found in *F. exsecta*. Thus, the increased amount of *n*-alkanes is associated with the task of foraging and is not age- or size-related.

Pogonomyrmex barbatus showed task-specific CHC profiles (Wagner et al. 1998). Foragers and patrollers were richer (≈5%) in *n*-alkanes than nest maintenance workers. The CHCs regulate the number of foragers (Greene and Gordon 2003), suggesting that *n*-alkanes mediate intraspecific communication in this species. This finding is supported by other studies of the intraspecific role of CHCs in ants (e.g., *F. japonica* Akino et al. 2004; *Linephithema humile* and *Aphaenogaster cockerelli* Greene and Gordon 2007; *F. exsecta* Martin et al. 2008a). Such studies have shown behavioral differences of ants towards glass beads treated with *n*-alkanes and *n*-alkane-free beads. However, these studies also detected that behavioral responses to other groups of hydrocarbons, e.g., alkenes, were even stronger than those to *n*-alkanes. This is consistent with the observations of Châline et al. (2005), who demonstrated that honeybees only poorly learned to discriminate between various *n*-alkanes when compared with alkenes of similar chain length. Therefore, ants may have to rely on large-scale differences in the amounts of *n*-alkanes to determine the task of an individual, whereas an increased sensitivity to alkenes allows small changes in alkene ratios to be used in nestmate recognition (Martin et al. 2008a).

Awareness of the proportion of foragers within a colony is information crucial for efficient colony organization (Holldöbler and Wilson 1990; Seeley 1995). Therefore,

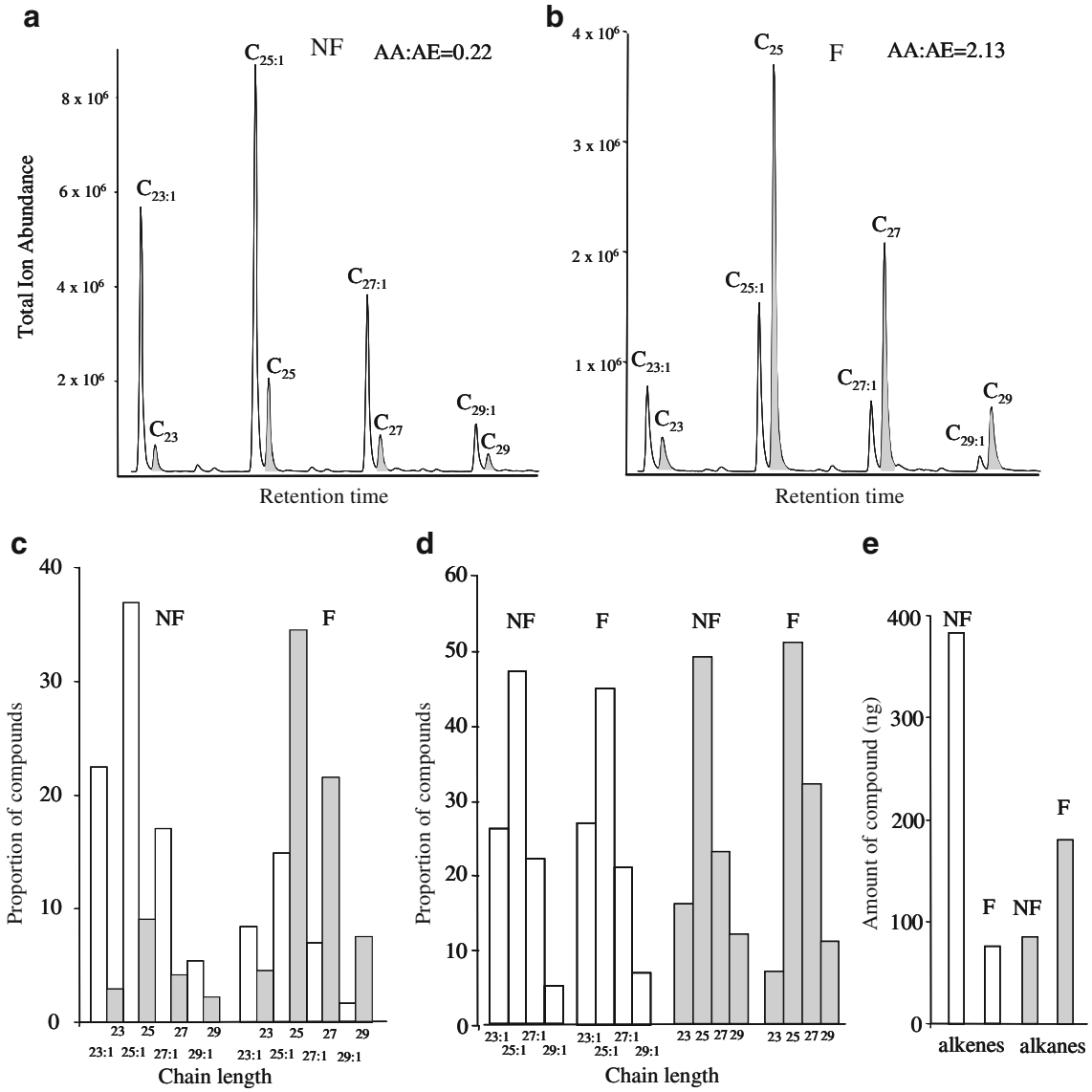

Fig. 2 A CHC profile of **a** a nonforager (*NF*) and **b** a forager (*F*) of the same colony (no. 2) that have very different *n*-alkane (*AA*)/alkene (*AE*) ratios. **c** The same nonforager and forager profiles remain distinct after normal standardization (i.e., proportions are calculated from total peak area). **d** However, when the proportions of each compound are calculated from the total peak area of those compounds belonging only to the same homologous series, the alkene proportions become stable despite **e** the two nestmates possessing very different amounts of alkenes. Throughout, *n*-alkanes (C_n) are shown as *gray peaks* or *bars*, whereas alkenes ($C_{n:1}$) are either *white peaks* or *bars*

the increased amounts of *n*-alkanes that help to protect foragers may have been co-opted in some species (e.g., *P. barbatus*) as a cue used by the colony thus modulating forager numbers (Greene and Gordon 2003). In this study, we showed that morphologically similar individuals can possess different chemical profiles, depending on their task. Pharaoh ants have morphologically identical ants that perform different behavioral tasks, e.g., "path finding"; such ants have chemically distinct profiles (Jackson et al. 2007). As the conveyance of information by chemical cues is a common mode of communication among insects, we expect many more of these "chemotypes" to exist, especially among social insects that possess a diverse array of chemical signals.

The presence of different proportions of two or more chemotypes within a sample has implications for the interpretation of results. It means that the CHC profiles can look very different among nestmates (Fig. 2a–c). The clear differences in amounts of *n*-alkanes between foragers and nonforagers allowed us to estimate the proportion of foragers sampled from the mound surface in ten *F. exsecta* colonies previously sampled (Martin et al. 2008a), and this ranged widely from 10% to 70%. This variation may be due to colony disturbance during ant collection since, once the colony is disturbed, many ants appear suddenly on the mound's surface. However, it means that the ratio of chemotypes within a sample can be highly variable (Fig. 2a,b), and this may generate misleading results (Martin and Drijfhout 2009). However, such variability (Fig. 2a–c) either disappears (alkenes) or is reduced greatly (*n*-alkanes) when the two groups of compounds are dissociated and then analyzed separately (Fig. 2d) or when ants are grouped correctly into foragers and nonforagers from the same nest. As expected for a nestmate signal, the colony-specific alkene ratio of two nestmates remained consistent irrespective of task [e.g., forager vs nonforager (Fig. 2d)], despite large differences in the amount of alkenes produced by each individual (Fig. 2a,b,e). Furthermore, intracolony variability in the alkene ratio is always lower than that of alkanes (Martin et al. 2008b), and there is only poor or no correlation between the total amounts of alkenes vs *n*-alkanes (this study) or between alkenes and *n*-alkanes of the same chain-length (Martin and Drijfhout 2009). This suggests that, for *F. exsecta* chemical communication, it is the distribution (ratios) of alkenes of different chain lengths (ratio of $C_{23:1}$ to $C_{25:1}$, $C_{25:1}$ to $C_{27:1}$, etc.) and not the total amount of alkenes that is important, whereas the opposite appears to be the case for the *n*-alkanes. Furthermore, Yamaoka (1990) found that, when a *Formica* sp. No. 5 (*Formica hayashi*) colony was made queen-less, the CHC profile of individual workers became highly variable. This increase in CHC variability was restricted, however, to the *n*-alkanes, whereas the amount of alkenes remained stable, thus maintaining the colony-specific profile. All these results suggest that the alkene chain length ratios remain stable among nestmates, whereas the *n*-alkanes are more labile, and as such, cannot be involved in nestmate, kin, or patriline recognition as has been suggested for honeybees (Arnold et al. 1996) and ants (Boomsma et al. 2003).

The fundamental functional differences and roles between *n*-alkanes (water-proofing) and alkenes (recognition signal) may help explain the consistent discrepancies found between the CHC profile and the profile within the postpharyngeal gland (PPG). The PPG is an exocrine gland unique to ants and is believed to be involved in pheromone communication (Soroker et al. 1995). In previous studies (Bagnères and Morgan 1991; Soroker et al. 1995; Akino et al. 2004), the proportions of *n*-alkanes were consistently higher on the cuticle than in the PPG gland. We suggest that *n*-alkanes also may be secreted directly onto the cuticle via various subepithelial glands known to exist in ants (Gobin et al. 2003), whereas compounds used in nestmate recognition, e.g., alkenes, may be sequestered and exchanged via the PPG.

In summary, the CHC profile of *F. exsecta* is composed of two distinct groups of components: an *n*-alkane part that varies among individuals depending on task, and a (*Z*)-9-alkene nestmate signal that remains stable across all colony members (Martin et al. 2008a). The task-driven profile changes associated with foraging are influenced by the environment, whereas nestmate- and species-recognition cues are genetically influenced since they remain stable irrespective of the environment changes either at a local [e.g., forager vs nonforagers (this study)] or geographical scale (Martin et al. 2008c). Thus, the CHC profile of *F. exsecta* is composed of both genetically (alkenes) and environmentally (*n*-alkanes) driven components.

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How Reliable is the Analysis of Complex Cuticular Hydrocarbon Profiles by Multivariate Statistical Methods?

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Abstract Numerous recent studies have correlated cuticular hydrocarbon profiles with a wide range of behaviors, particularly in social insects. These findings are wholly or partly based on multivariate statistical methods such as discriminate analysis (DA) or principal component analysis (PCA). However, these methods often provide limited insight into the biological processes that generate the small differences usually detected. This may be a consequence of variability in the system due to inadequate sample sizes and the assumption that all compounds are independent. A fundamental problem is that these methods combine rather than separate the effects of signal components. By using cuticular hydrocarbon data from previous social insect studies, we showed that: (1) in 13 species of *Formica* ants and seven species of *Vespa* hornets, at least one group of hydrocarbons in each species was highly ($r^2>0.8$) correlated, indicating that all compounds are not independent; (2) DA was better at group separation that PCA; (3) the relationships between colonies (chemical distance) were unstable and sensitive to variability in the system; and (4) minor compounds had a disproportionately large effect on the analysis. All these factors, along with sample size, need to be considered in the future analysis of complex chemical profiles.

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Introduction

During the past 15 years, there has been an explosion of studies correlating cuticular and glandular chemical profiles with a wide range of behaviors across a broad range of species. Social insects (ants, bees, termites, and wasps) in particular have been shown to possess complex chemical profiles correlated with complex behaviors, and these have been the focus of numerous studies (see reviews by Lenoir et al. 2001; Howard and Blomquist 2005). Due to the widespread availability of gas chromatography (GC) and GC-mass spectrometry (MS) machines, the vast majority of these studies have focused on cuticular hydrocarbons (CHC). CHC occur on the external surfaces of all insects (Lockey 1988) and have been implicated in recognition at various levels of organization mediating many key behaviors, such as aggression and altruism among species, nestmates, and kin. The majority of these findings have been based on multivariate statistical methods such as discriminate (DA) (e.g., Dapporto 2007) or principal component analysis (PCA) (e.g., Steiner et al. 2007). More novel methods that use genetic algorithms (Lavine et al. 2003), self-organizing maps (Steiner et al. 2002), and neural networks (Bagnéres et al. 1998) have been used with varying degrees of success.

CHC profiles often consist of 20–100 individual compounds, and this high degree of data dimensionality makes it difficult to interpret the relationships among compounds and differences among individuals or colonies without reducing data to fewer dimensions. This is compounded by the fact that, with the exception of sex pheromones, very

few compounds or profile components have been identified that have been linked directly with the observed behavior, e.g., nest-mate discrimination (Akino et al. 2004; Martin et al. 2008a). Furthermore, many signals used for species, colony, kin, fertility, or task recognition appear to be encoded by using the same shared components of the CHC profile (Denis et al. 2007). This makes isolation of the various signal components difficult, and we are only just starting to understand how these various signals are encoded (Martin et al. 2008a, b, c).

The advantage of multivariate statistical methods is that highly complex data sets are reduced to a single data point, which can be visualized in relation to other data points and interpreted by the investigator in light of the observed behavior. This is a powerful feature of these methods because they can detect small differences among groups, which are particularly useful for chemical ecologists studying the complex CHC patterns of social insects. However, these methods often have provided limited insight into the biological processes that generate the differences. Most often, isolated compounds subsequently have failed to produce the expected behavioral response when bioassayed (G. Jones and T. Monnin, personal communication). The central problem is that multivariate statistical methods combine rather than separate out the effects of signal components, which can produce unstable results. As recognition signals in ants, and probably many other insects, are known to consist of several compounds, those ratios are stable at a certain level, e.g., colony (Akino et al. 2004; Martin et al. 2008a, b) or species (Martin et al. 2008c). DA then is a good method to demonstrate group differences. However, this may be appropriate only if other types of variation that originate from other signals or extraneous sources are eliminated, since the strength of DA and PCA can also be the weakness. That is, the results of DA/PCA reflect *all* the variation in the system, and it is becoming more apparent (Greene and Gordon 2003; Martin et al. 2008a) that various signals are encoded only in part, rather than all, of the profile.

In almost all studies, each peak (compound) in a profile is treated as an independent variable. Normally, the number of samples per group must exceed the number of variables (predictors or peaks). This basic statistical rule is overlooked routinely by both investigators and reviewers. In fact, when using multiple regression methods, the ratio of observations to independent variables should not fall below five (Bartlett et al. 2001), with ten observations to each independent variable being optimal (Halinski and Feldt 1970; Miller and Kunce 1973). This is especially critical when using continuous data, like that generated in CHC studies. It is common that ratios lower than one are used, and so there is a risk of overfitting, meaning that results cannot be generalized (Hair et al. 1995). The same ratio considerations apply to PCA, since an increase in sample size decreases the level at which an item loading on a factor becomes significant. Therefore, an analysis should not be done with less than 100 observations (Hair et al. 1995).

It is often the case that prior knowledge of the data classification is known (e.g., colony type), and in those cases, DA should be preferred over PCA, as this method guarantees the optimal separation among the clusters. Indeed, where the data are prestructured, the principal components are evidently known. In fact, PCA does not consider the cluster structure at all, and there is no guarantee that the resulting projection will separate clusters (Sanguinetti 2008).

In general, each of the compounds found in a CHC profile is assumed to be unrelated to other compounds. However, it has long been known (Lockey 1988), but overlooked, that the majority of CHC (alkanes, alkenes, and monomethylalkanes) belong to homologous series in which the chain length increases by two carbons (e.g., C_{25}, C_{27}, C_{29}). The positions of double bonds or methyl groups are also known to remain constant (e.g., $3MeC_{25}$, $3MeC_{27}$, $3MeC_{29}$). There is, thus, a strong probability that compounds within these homologous series may be correlated, as they share common biosynthetic pathways (Morgan 2004). This leads to multicollinearity problems and unstable results if multivariate methods are used (Field 2005).

The aim of this study was to use data from previous social insect studies to investigate: (1) if high levels of correlation among compounds within the CHC profiles exist and (2) how changing the variability of the signals both within a group and among groups affects the resulting DA and PCA analysis, by using a two-signal model based on the ant *Formica exsecta*. The nest-mate signal in two closely related species of ants *Formica japonica* (Akino et al. 2004) and *F. exsecta* (Martin et al. 2008a) is based on the unique colony distribution of (Z)-9-alkenes. Both these species have a simple CHC profile that consists of a single homologous alkane and alkene series. Intracolony variation in the amount of alkanes is variable since foragers have higher amounts of alkanes than nonforagers (Martin and Drijfhout 2009). This means that the two-signal model is realistic; (3) the effect of minor compounds on the DA analysis of actual *F. exsecta* data to test how well the known colony-specific compounds are correctly identified in the structure matrix.

Methods and Materials

Relationship among Compounds From 2005 to 2007, large CHC data sets have been amassed for 13 species of *Formica* ants predominantly from Finland (565 individuals) (Martin et al. 2008c) and from seven species of Japanese

hornets (*Vespa*) (142 individuals) (Martin et al. 2008d). For each species (Table 1), at least 15 individuals from three different colonies were analyzed. The data for each individual exists in three forms: (1) the relative amounts of each compound reported as the number of ions contained beneath the peak, (2) the proportion (%) of each compound with respect to the total amount ions detected from those individual, and (3) the transformed proportions from using the method of Reyment (1989) and Aitchison (1986):

$$Zi,j = \mathrm{Ln}[Yi,j/g(Yj)]$$

where Yi,j is the area of peak i for the individual j, $g(Yj)$ is the geometric mean of the areas of all peaks for individual j, and Zi,j is the transformed area of peak i for individual j. These three data blocks were arranged in Microsoft® Excel spreadsheets so that correlations among compounds could be compared across individuals from the same colony or species by plotting the values on a scatter graph and using the linear trend line function to calculate the r^2 value. We compared the predominant compounds that occurred within each species.

Table 1 Correlations (r^2) between relative amounts of two consecutive hydrocarbons within a homologous series from *Formica* ants and *Vespa* hornets

Compound	r^2	Species
Alkanes		
C_{21} to C_{25}	>0.84	*F. cinerea*
C_{23} to C_{31}	>0.91	*F. aquilonia*
C_{25} to C_{31}	>0.97	*F. lugubris*
C_{23} to C_{33}	>0.90	*F. polyctena*
C_{23} to C_{33}	>0.88	*F. pratensis*
C_{23} to C_{29}	>0.80	*F. candida*
C_{27} to C_{33}	>0.92	*F. uralensis*
C_{27} to C_{33}	>0.93	*F. truncorum*
C_{23} to C_{27}	>0.90	*F. fusca*
C_{25} to C_{31}	>0.98	*V. mandarina*
C_{21} to C_{25}	>0.84	*V. dybowskii*
C_{27} to C_{33}	>0.89	*V. ducalis*
C_{25} to C_{27}	>0.88	*V. simillima*
Alkenes		
$C_{23:1}$ to $C_{31:1}$	>0.94	*F. candida*
$C_{23:1}$ to $C_{29:1}$	>0.91	*F. lemani*
$C_{23:1}$ to $C_{29:1}$	>0.95	*F. exsecta*
$C_{27:1}$ to $C_{31:1}$	>0.82	*F. lugubris*
$C_{25:1}$ to $C_{27:1}$	>0.92	*F. sanguinea*
$C_{27:1}$ to $C_{29:1}$	>0.98	*V. simillima*
$C_{27:2}$ to $C_{29:2}$	>0.98	*F. candida*
Mono-methyl alkanes		
3-Me C_{23} to C_{29}	>0.91	*V. crabro*
3-Me C_{27} to C_{29}	>0.98	*V. affinis*
3-Me C_{23} to C_{25}	>0.90	*F. fusca*
3-Me C_{23} to C_{29}	>0.94	*F. lemani*
7-Me C_{21} to C_{25}	>0.87	*F. cinerea*
9,11,13-Me C_{23} to C_{31}	>0.83	*F. sanguinea*
9,11,13-Me C_{25} to C_{35}	>0.93	*F. aquilonia*
9,11,13-Me C_{23} to C_{29}	>0.90	*V. simillima*
13-Me C_{26} to C_{30}	>0.81	*F. rufa*
Di-methyl alkanes		
7,15 diMe C_{21} to C_{25}	>0.88	*F. cinerea*
11,15 diMe C_{27} to C_{29}	>0.98	*V. analis*
10,18 diMe C_{30} to C_{34}	>0.93	*F. aquilonia*

For example, C_{21} to C_{25} indicates that C_{21} vs. C_{23} and C_{23} vs. C_{25} both had r^2 values greater than 0.84.

Two-Signal Model We modeled a two- (nest-mate and task) signal system based on the ant *F. exsecta*. We constructed five groups (colonies), each of which contained 10 individuals (ants) that had a profile composed of two independent signals (S_1=nest-mate and S_2=task). Each signal was composed of four variables (compounds), whose ratios can be correlated (constant within a group) or not correlated (highly variable). During the simulation, five unique group profiles (distributions) were used. An example of how two different groups with the least and most amount of intragroup signal variation were constructed is shown in Fig. 1. When the intra- or intercolony signal was constant, we added a small (<3%) amount of variation to the proportion of each compound because multivariate statistical analysis is obviously not possible if no variation in the data exists.

For each model run, we constructed 50 individual (ant) profiles that were grouped into their five respective groups (colonies). For each individual, the values of the eight compounds that make up the two signals were combined, and the proportion of each compound was recalculated since proportions are always calculated by using all peaks present. These data were geomean-log transformed and analyzed by using both discriminant analysis (DA) function in SPSS v.14 and principal components and classification analysis (PCA) function in Statistica v. 6, the first and second factors plotted. The DA used a grouping variable where all independents were entered together with a within-group covariance matrix. The PCA analysis was based on correlations and variances computed as $SS/(N-1)$, and no grouping variable was used. Different combinations of within-group (large vs. small) and between-group variation in the signals were investigated. Additionally, it is known that the relationship between two different signals does not always remain constant across all members of a group (Martin et al. 2008a), so we also measured the effect on the DA and PCA analysis when the ratios of the two signals

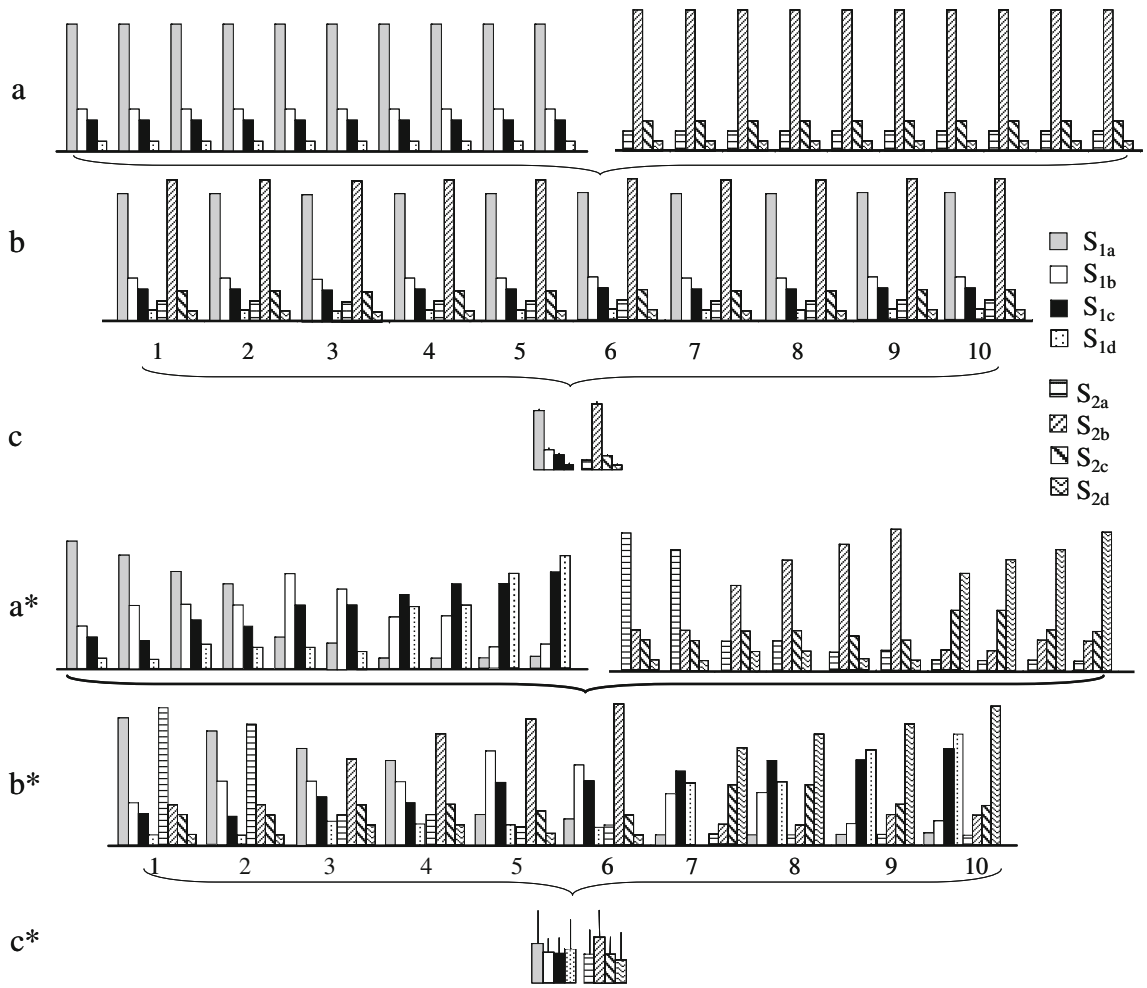


Fig. 1 The composition of two groups in which the two signals are either constant (**c**) or highly variable (**c***). Each group member (**b**–**b***) has a profile containing two signals S_1 and S_2 (**a**–**a***) that are in turn composed of four compounds ($S_{1a–d}$, $S_{2a–d}$). Each group (**c**) is composed of 10 individuals that have a similar (**a**, **b**, **c**) or different (**a***, **b***, **c***) profiles. The four compounds of each signal will be highly correlated in (**c**) and not correlated in (**c***)

(S_1 and S_2) remained constant (1:1) or varied (1:0.3–3) within each group. This is extraneous variation since it is totally dependant on the composition of the group sampled and arises from sampling morphologically identical individuals that represent different chemo-types. For example, individuals engaged in some task or with a specific physiological state may have part of a profile that is chemically distinct (Jackson et al. 2007).

Effects of Minor Compounds By using real data from five *F. exsecta* colonies that were analyzed in the same way as above, we compared the DA results when eight major CHC were analyzed, with results when six minor components were added. We then artificially reduced within-group variation in the minor compounds from c. 10% to c. 3% and investigated the effect on group separation under different conditions. We also investigated the structure matrices to see how well known key compounds were identified.

Results

Relationship among Compounds In all 13 species of *Formica* ants and seven species of *Vespa* hornets, at least one group of compounds within a homologous series were highly (r^2>0.8) correlated. Furthermore, these high correlations could occur in any type of homologous series (Table 1). Correlations may sometimes extend across homologous series among compounds with the same chain length, but this feature of the CHC profile is highly variable (Table 2).

The relative amounts of $C_{23:1}$ and $C_{25:1}$ in five different *F. exsecta* colonies was always highly correlated. This correlation persisted when the amounts were converted into proportions after the values were geomean-log transformed (Table 3).

Two-Signal Model In most simulations, both DA and PCA successfully separated out the five colonies, although, as

Table 2 Correlation (r^2) between major compounds with the same chain length but belonging to different homologous series in seven different species of *Formica* ants from Finland and one UK population

	F. truncorum	*F. rufa*	*F. uralensis*	*F. pratensis*	*F. lugubris* (UK)	*F. lugubris*	*F. polyctena*	*F. aquilonia*
Alkanes (C_*) vs. alkenes ($C_{*:1}$)	0.02	0.04	**0.81**	0.50	0.45	0.07	0.01	0.17
Alkanes (C_*) vs. (meC_*) mono-methyls	0.10	0.15	**0.82**	0.16	0.50	0.05	0.14	0.46
Alkenes ($C_{*:1}$) vs. (meC_*) mono-methyls	0.28	0.30	**0.93**	0.69	**0.91**	**0.92**	0.08	0.18
Monomethyls (meC_*) vs. ($dimeC_*$) di-methyls	**0.99**	0.51	**0.97**	0.62	**0.92**	**0.92**	0.59	**0.97**

The highly correlated values are given in bold.

expected, group differences were consistently better resolved by DA (Fig. 2). DA was able to clearly separate the five groups even when the group differences were small (Fig. 2b), although resolution is lost when extraneous variation was introduced (i.e., between the two signals). However, both methods were unable to distinguish between two or one group-specific signals (compare Fig. 2a and c) or show whether the differences between the group signals were large or small (compare Fig. 2a and b). The entire removal of one of the signals (S_1 or S_2) produced a similar but not identical result, as shown in Fig. 2a–c. As the total amount of signal variation increased, the DA factor scales generally decreased, but not in a consistent way. Also, the relationships among colonies (chemical distance) were unstable as they changed depending on the amount of real or extraneous variability in the system and the method used. For example, the chemical distance between groups 1 and 4 is small when analyzed by DA but large when analyzed by PCA (Fig. 2a).

Effects of Minor Compounds The DA separated out the five different colonies only when major compounds (Fig. 3a) or when all compounds (major and minor) were used (Fig. 3b). However, when the within-colony variation of only the minor compounds was reduced, better separation was obtained (Fig. 3c). When the between-colony variation in all compounds was reduced to <3% for all compounds, no separation occurred (Fig. 3d). The addition of natural levels of variation in only the six minor compounds, however, resulted in colony separation (Fig. 3e). This effect illustrates the power of minor compounds on the analysis. This is supported by the structure matrix data that consistently fail to correctly identify the major alkenes that are known to encode (Martin et al. 2008a) the nest-mate recognition system in this species.

Discussion

We have shown that DA and, to a lesser extent, PCA are good at separating group differences, even when these differences are small (see Fig. 2b). However, they provide limited information about the actual number of signals present. For example, the results are indistinguishable whether one or two distinct signals are present (compare Fig. 2a and c).

Our investigation of *Formica* ant and *Vespa* hornet data illustrates that multicollinearity is a common property of CHC profiles, and it indicates that this is probably a widespread phenomenon. This suggests that compounds share common biosynthetic pathways, particularly in the case of by-products that are likely to be closely linked to the main product. Importantly, where compounds are highly correlated ($r^2>0.8$) within a colony, population, or species, that series of compounds can be treated as a single variable. This approach greatly reduces the data dimensionality of the profile and helps focus in on key compounds, or more correctly, related groups of compounds. It also reduces the requisite number of predictors, meaning that fewer samples are needed. However, the number of samples still must exceed the number of predictors by at least a ratio of five to one (Halinski and Feldt 1970; Miller and Kunce 1973; Bartlett et al. 2001) unless evidence is provided to the contrary. In the future, studies need to be conducted on species where sample size is not a limiting factor, which, in

Table 3 The correlation (r^2) between $C_{23:1}$ and $C_{25:1}$ from five *F. exsecta* colonies where 10 workers were analyzed from each colony

	F. exsecta				
	Col-22	Col-35	Col-40	Col-56	Col-60
Relative amount (ion count)	0.99	0.99	0.99	0.99	0.99
Proportions	0.93	0.93	0.88	0.97	0.91
Transformed proportions	0.99	0.95	0.90	0.98	0.94

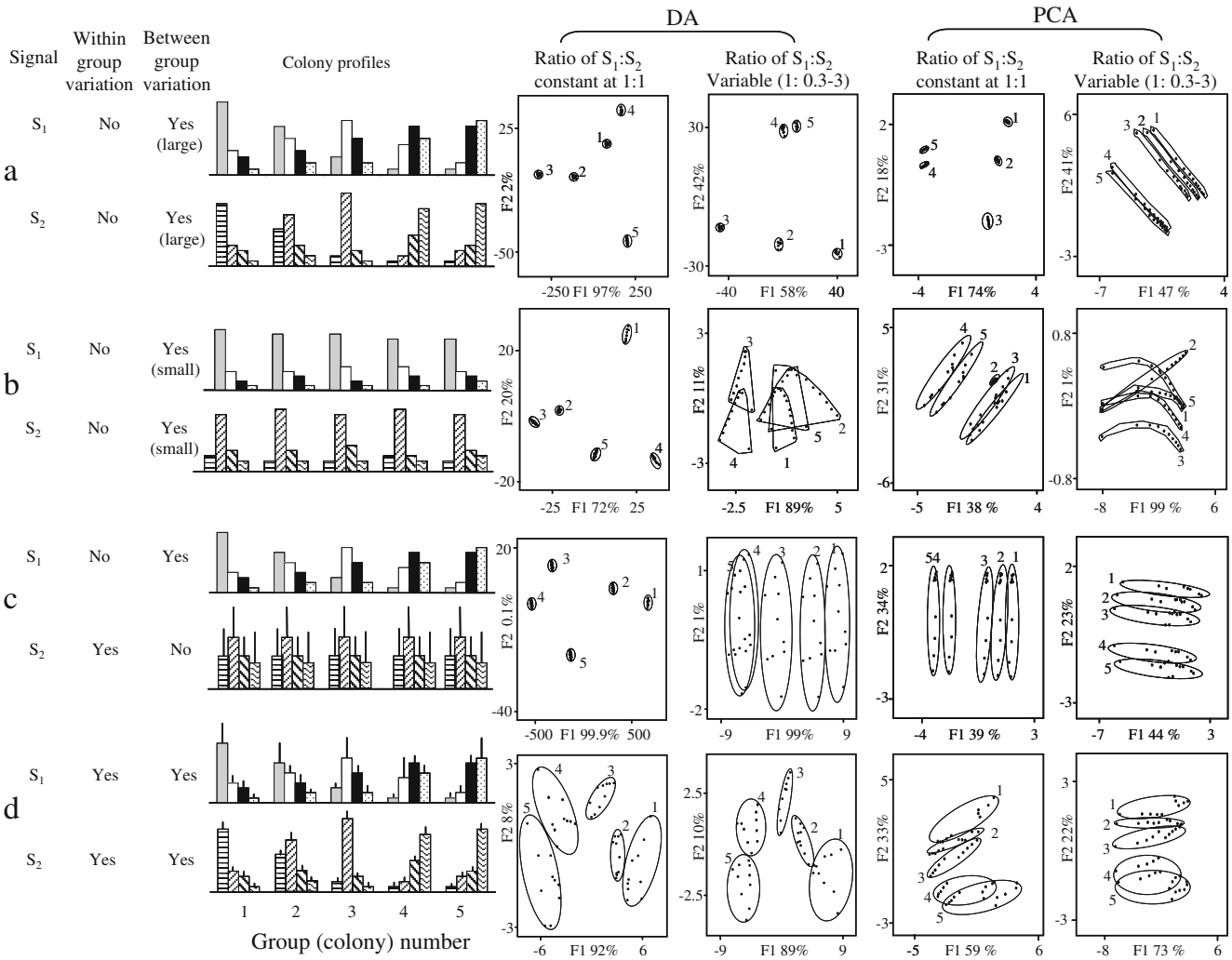
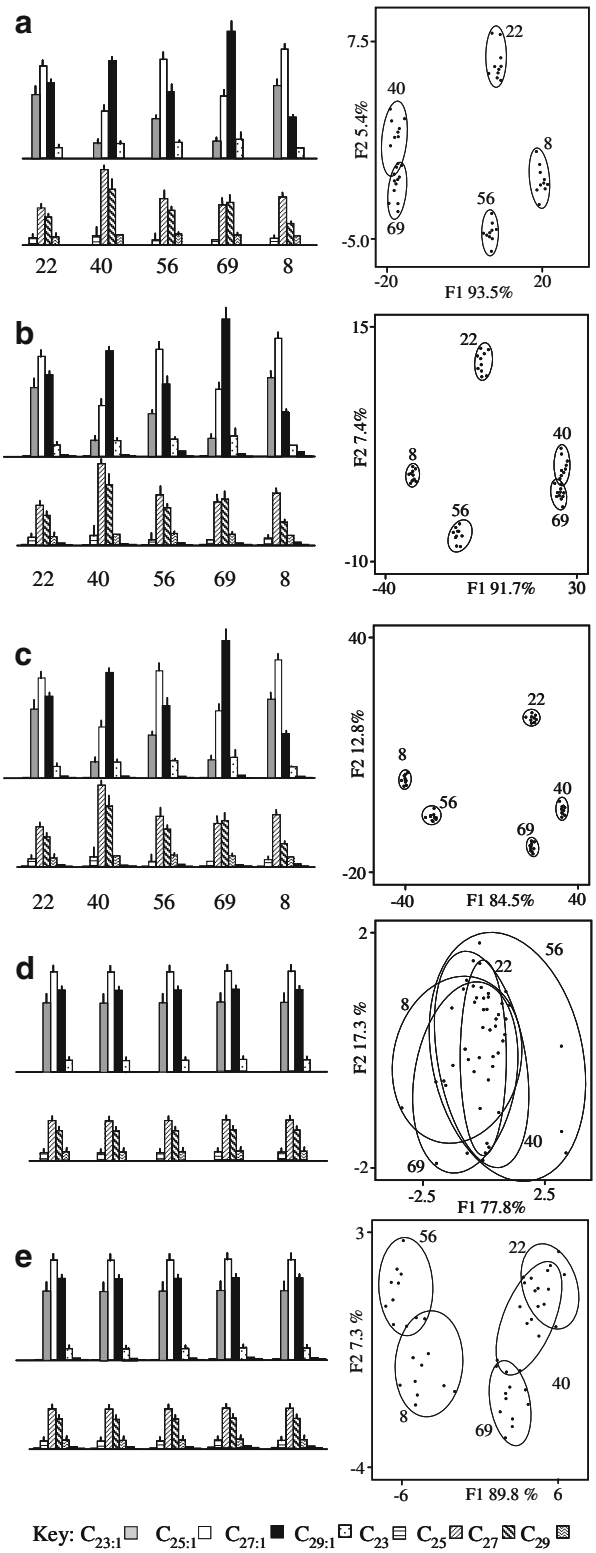


Fig. 2 Effect of varying the amount and type of variation of two signals on the results produced by DA and PCA. For each simulation (**a**–**d**), the histograms illustrate the five group signals, with the *error bars* representing the SD. Only error bars are shown were the error is >3%. The resulting combined plots obtained by DA and PCA are shown when the ratio of the signals (S_1:S_2) was both variable and constant. All 10 data points for each group are enclosed within a *line*

the case of many social insect species, should not be a problem. The downside of multicollinearity is that it violates the equal intraclass covariance assumption that underpins DA and PCA (Sanguinetti 2008), thus making the results of multivariate statistical methods unstable and sensitive to small changes. Thus, PCA/DA is sensitive to small changes, especially background noise. As we have shown, the addition of minor compounds, and their variation (Fig. 3) or variation between signals (Fig. 2), alters both the scale and the intergroup relationships, thus making interpretation of the results unstable. Furthermore, researchers also must be aware that increased collinearity among compounds raises the chances of encountering type II error, and the transformation of the data does not remove this multicollinearity problem (Table 3).

When compounds cannot be integrated, due to their absence or because they lie below the instrument detection limit, then a small nominal value must be used during the data transformation since a geomean cannot be calculated if zeros are present (Steiner et al. 2007). Therefore, potential qualitative differences are not reflected in the analysis, and these transformed nominal values will reduce the amount of variation among minor compounds and possibly increase the chemical distance between existing groups (see Fig. 3). Chemical distances among groups are highly unstable as they are sensitive to the amount of variation in the system and even the method used (Fig. 2a). They are only really robust when only one known signal is being analyzed.

These potential problems should be considered before employing multivariate statistical methods that are often systematically used. Only by separating out the various signals can the signals contained within the CHC profile be decoded. It is becoming apparent that different species use different methods to encode recognition signals



Structure Matrix

	Function 1	2	3	4
C_{29}	-.010	-.091*	.060	.053
C_{23}	-.005	.360	.784*	.101
C_{25}	-.021	-.025	.465*	.330
$C_{27:1}$	-.089	.056	-.392*	-.226
$C_{29:1}$	-.057	-.182	-.291*	-.202
C_{27}	-.069	-.034	.085	.428*
$C_{23:1}$	.182	.137	-.233	-.356*
$C_{25:1}$	.078	-.047	-.258	-.270*

	Function 1	2	3	4
$C_{21:1}$	.097	.279*	.103	-.246
C_{21}	.041	.252*	.040	-.122
$C_{23:1}$	-.110	.154*	.100	.147
C_{23}	.010	.134	-.747*	.401
C_{25}	.012	-.044	-.449*	.311
$C_{31:1}$	-.082	-.279	.286*	.076
$C_{27:1}$	.060	-.002	.198*	.177
$C_{25:1}$	-.050	.013	.157*	.125
$C_{33:1}$	-.045	-.228	.119	-.560*
C_{33}	-.027	-.052	.092	-.417*
C_{27}	.046	-.062	-.159	.405*
C_{31}	-.022	-.010	.036	-.234*
C_{29}	.005	-.068	-.106	.217*
$C_{29:1}$	.034	-.125	.171	.212*

	Function 1	2	3	4
$C_{33:1}$	-.231	-.371*	.047	-.080
C_{21}	.127	.313*	-.285	-.133
$C_{23:1}$	-.068	.223*	-.025	.115
C_{33}	-.116	-.073	-.374*	.142
C_{31}	-.057	-.106	-.231*	.072
$C_{31:1}$	-.151	-.153	.217*	.108
C_{27}	.029	-.049	.107*	-.095
C_{23}	.035	.097	.080	-.629*
$C_{21:1}$	.194	.099	-.295	.520*
C_{25}	.010	-.014	.094	-.321*
$C_{27:1}$	.055	-.053	.033	.210*
$C_{25:1}$	-.035	.072	.019	.154*
$C_{29:1}$	.019	-.084	.082	.151*
C_{29}	.001	-.021	.061	-.065*

	Function 1	2	3	4
C_{27}	.556*	.099	.406	-.211
C_{25}	-.011	.359	.673*	-.020
C_{23}	.151	.356	.526*	.494
$C_{27:1}$	.131	.276	.481*	.404
C_{29}	.120	.241	.301*	.214
$C_{29:1}$	.093	.128	.354	.513*
$C_{23:1}$	.121	.342	.390	.452*
$C_{25:1}$	.122	.360	.287	.376*

	Function 1	2	3	4
$C_{33:1}$	-.250	-.398*	.046	-.233
C_{27}	-.030	.223	.286*	.230
$C_{21:1}$	.443	-.120	.284	-.474*
C_{33}	-.083	-.159	-.226	-.461*
C_{25}	-.001	.036	-.096	.424*
C_{23}	.000	.097	.022	.407*
$C_{31:1}$	-.225	.012	.095	-.367*
C_{21}	.180	.078	-.203	-.364*
$C_{27:1}$	-.002	.078	.026	.350*
$C_{23:1}$	.003	.086	.007	.338*
$C_{25:1}$	.004	.093	.001	.294*
$C_{29:1}$	-.001	.042	.045	.245*
C_{29}	-.001	.075	.020	.244*
C_{31}	-.039	-.034	-.192	-.204*

Fig. 3 The effect of minor compounds on the analysis is illustrated using real data from five *F. exsecta* colonies. **a** Uses only the eight major compounds; **b** uses both the eight major and six minor components, i.e., with values <2%, which vary naturally; **c** uses the same compounds as **b**, but the within-group variation of the six minor compounds is reduced; **d** between-colony variation is low in all compounds, whereas in **e**, between-colony variation is present only in the minor compounds. Each *panel* illustrates the actual alkane and alkene colony profiles, the resulting DA, and the structure matrix, which gives the pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions. The major compounds known to be used in nest-mate recognition in this species (Martin et al. 2008a) are shown in *bold*. *Asterisks* mark the largest absolute correlation between each compound and any discriminant function

(Martin et al. 2008b). Currently, there is no suitable method that will easily reveal these mechanisms, and this includes DA because it produces no reliable information about the composition of the signal (Fig. 3). Study of the various parts of the profile is more biologically relevant since different groups of hydrocarbons are perceived differently, e.g., alkanes vs. alkenes (Chaline et al. 2005; Dani et al. 2005). This means that the profile perceived by the insect and that produced by the GC-MS are not congruent. Simple regression analysis has begun to reveal

(Martin et al. 2008b) that apparently complex CHC profiles are in fact a mixture of correlated and non-correlated compounds. The study of their relationships across many individuals and the use of methods such as electroantennagrams are starting to help us decode the signals contained within these profiles.

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Milieu-Dependent Pro- and Antioxidant Activity of Juglone May Explain Linear and Nonlinear Effects on Seedling Development

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Abstract Juglone, 5-hydroxy-1,4-naphthoquinone, is known for its wide range of biological activities. It has been suggested that juglone's excellent redox cycling properties contribute to this reputation. Many biological activities are nonlinear with low concentrations exerting stimulating effects, whereas only higher concentrations cause inhibition. Here, we corroborate studies on the nematode *Caenorhabditis elegans* that point out hormetic effects by showing that juglone may cause a nonlinear effect on postgerminative shoot and root growth of *Sinapis alba*. This effect was only significantly visible, however, when seedlings were stressed with methanol. Classic and modified versions of the deoxyribose assay were applied successfully to characterize antioxidative (purposeful generation of hydroxyl radicals) and prooxidative (no purposeful generation of hydroxyl radicals) activities. Variants of the assay with and without the addition of the iron chelator EDTA showed that the antioxidant activity is independent on chelation of iron ions by juglone; by contrast, the strength of the prooxidative activity depended on the chelation of iron ions by juglone. The hormetic effects of lower concentrations on germination of *Sinapis alba*, thus, may be caused by the antioxidant activities of this compound, which are especially effective when the test organism is subjected to higher oxidative challenge. The present study suggests that pronounced prooxidative activities, which are considerably accelerated by chelation of iron ions, may contribute to the toxic effects of juglone at higher concentrations.

Keywords Naphthoquinones · *Sinapis alba* · Inhibition · Hormesis · ROS · Fenton reaction · Chelation of iron · Stress

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Introduction

Although the need for greater use of modern technology and additional research on allelopathic mechanisms has been articulated (Romeo 2000), this area remains somewhat neglected relative to traditional phytotoxicity experiments. A few recent studies include those on: juglone effects on root plasma membrane H^+ ATPase activity and root water uptake (Hejl and Koster 2004); the use of polydimethylsiloxane materials to quantify levels of the photosynthesis inhibitor sorgoleone in the rhizosphere of sorghum plants (Weidenhamer 2005); the critical evaluation of the contribution of (±)-catechin to invasion success of spotted knapweed (Blair et al. 2005, 2006); degradation of soluble phenolics by ectomycorrhizal fungi, thus controlling species interactions in black spruce stands (Zeng and Mallik 2006); selective phytotoxicity of L-DOPA because of differential polyphenol oxidase activity in barnyard grass and lettuce, thus mediating reactive oxygen species and/or free radical species (Hachinohe and Matsumoto 2007); and differential gene expression in rice under low nitrogen due to stronger activation of genes that function in synthesis of allelochemicals (Song et al. 2008).

Often, we do not pay much attention to studies carried out many decades ago, simply because, compared to current standards, the methodologies have dramatically changed. Hans Molisch is generally regarded as the person coining

the term allelopathy. In his experiments, he used the odor of apple peels, mainly comprised of ethylene, as a model system to explore effects of volatile emissions on the development of a range of plant species. He reported the results in his famous booklet "Der Einfluß der Pflanze auf die andere, Allelopathie" (the effect of one plant on another, allelopathy); there also exists an English translation (Molisch 2002). Molisch's observations contain some fundamental insights, which are still under discussion today. In the summary, Molisch notes: "If seedlings are only exposed to the gas emitted by apples a relatively short time, then it becomes evident, that not an inhibition of longitudinal growth occurs but a very pronounced stimulation. Exposure to apple odor for one to five hours caused an unambiguous stimulation, whereas 24 hours caused a slight inhibition that became more pronounced with ongoing exposure". He also provides an interpretation: "Here, the often observed rule is confirmed that poisons and irritating compounds are harmful in higher concentrations, but stimulate in diluted form". The last sentence is especially notable, because Molisch (1937) already acknowledges a nonlinear mode of action for allelochemicals. In many instances, the response of living organisms to abiotic or biotic effectors is nonlinear, i.e., at low dosages the response is opposite to that of high ones; high doses inhibit growth and low doses stimulate growth. The latter phenomenon is also known by the term hormesis (Calabrese et al. 2007). Although it has been known for a long time, it has been largely ignored because nobody could explain it (Stebbing, 1982). Allelopathic studies that address the phenomenon of hormesis are infrequent, and then the phenomenon is addressed usually only peripherally (Romagni et al. 2000; Belz 2008). However, it has been focused on recently in ecological modeling of allelopathy (Sinkkonen 2007). In their recent survey of hormesis in plant biology, Calabrese and Blain (2009) note that allelopathy is a rapidly developing area in plant chemical biology in which hormetic responses of chemical exudates from plant roots are to be considered.

Juglone, 5-hydroxy-1,4-naphthoquinone, is classified as a strong redox cycler with high potential to react with oxygen and its reactive species. Thereby, it interferes with vital cell processes such as photosynthesis, respiration, cell division, and membrane transport (Bertin et al. 2003; Tomilov et al. 2006). Among others, one of the noted harmful effects is the alleged role in the allelopathy of walnut trees (Jose 2002). In an attempt to optimize simple tube assay procedures, thus allowing us to explore the potential redox reactions of allelochemicals, modified variants of the deoxyribose assay were used. This assay was developed as a simple screening method for hydroxyl radical scavengers (Aruoma et al. 1987; Halliwell et al. 1987). The recognized function of juglone as a redox cycler distinguishes this allelochemical as a suitable model for exploring the combined applicability of simple tube assays with biological assays to obtain further insights about the milieu-dependent reactivity of candidate molecules. In this study, the effects of juglone on seedling development of *Sinapis alba* served as the biological assay. In consideration of the proposed role of reactive oxygen species (ROS) in the mode of action of juglone, we used an agar- and filter paper-based growth assay to simulate low and high level of oxidative stress caused by variable additions of methanol (0.2 and 10%, *v/v*, respectively) to the medium. We expected to detect nonlinear effects (hormesis) at higher methanol levels. Hormetic abilities previously have been reported for effects of juglone on the genetic model nematode *Caenorhabditis elegans*; these effects became especially evident when *C. elegans* was pretreated with low dosages of heat, hyperbaric oxygen, or even juglone itself, and then subjected to subsequent stresses of the original or one of the other stressors (Cypser and Johnson 2002).

Materials and Methods

Chemicals All chemicals were obtained from Sigma Aldrich Inc. (St. Louis, MO, USA) unless otherwise stated; water had Milli-Q quality.

Antioxidative Assays Procedures followed those described by Halliwell et al. (1987) and Aruoma (1994) for the deoxyribose assay. Juglone (Fluka, Buchs, Switzerland) was dissolved in an aqueous KH_2PO_4/KOH buffer solution (50 mM, pH 7.4) to yield final concentrations of 2–500 µM. To 125 µl of this solution, 25 µl of a 10.4 mM 2-deoxy-D-ribose solution in the same buffer system and 50 µl of an aqueous solution of $FeCl_3$ (50 µM) were added. In one series of the assay, those 50 µl contained 52 µM EDTA in buffer, and in the other series, only buffer was added. To start the Fenton reaction, various reactants dissolved in the above mentioned buffer systems or in water were added: 25 µl 10.0 mM aqueous solution of H_2O_2 and 25 µl 1.0 mM ascorbic acid in buffer. Standard 1.5 ml sample vials (La-Pha-Pack, Werner Reifferscheidt GmbH, Langerwehe, Germany) were used as reaction vials. The mixture was vortexed and incubated at 27°C for 60 min. Thereafter, 10 µl of 2.5% ethanolic butylated hydroxytoluene solution followed by 250 µl of 1.0% 2-thiobarbituric acid dissolved in 3% trichloroacetic acid were added to each vial to detect malonyldialdehyde, the decomposition product of 2-deoxy-D-ribose caused by the attack of hydroxyl radicals. The vials were vortexed and heated in a water bath at 85°C for 30 min. The reaction was stopped by transferring the vials into an ice water bath for 3 min. To extract the reaction product of malonyldialdehyde

and thiobarbituric acid, 600 µl of *n*-butanol was added and the mixture was rigorously vortexed. The butanol layers of the vials, each 350 µl, were pipetted into flat-bottomed 96-well plates (Greiner, Kremsmünster, Austria), and the absorbance was determined with a microplate reader (Tecan Infinite M200, Männedorf, Switzerland) at 532 nm. Assays were performed in triplicate. Reaction mixtures lacking the test compound served as negative controls. Blank was reactive mixture without 2-deoxy-D-ribose.

Prooxidative Assays This modification of the deoxyribose assay was carried out without the addition of H_2O_2 and ascorbic acid. Reactions of juglone depended strongly on the dissolving of oxygen into the liquid. Thus, scoring was performed after 16 h.

Postgermination Growth Assays (Low Stress Scenario) Seeds of *S. alba* L. were obtained from B and T World Seeds (Paguignan, France) and surface sterilized (1 min in 70% EtOH). Petri dishes (9 cm diam, Greiner Bio-One, Kremsmünster, Austria) were prepared with solidified Murashige and Skoog (MS) basal medium and spiked with a methanolic solution of juglone to provide concentrations of 6–200 µg ml^{-1} juglone in the agar medium. Controls were spiked with methanol alone (0.2% *v*/*v*). Surface-sterilized seeds were transferred to the agar medium with sterilized forceps. Petri dishes were incubated at 25±2°C in dark. After 5 days, control and treated seedlings were photographed with an Olympus D500 digital camera equipped with a 35 mm macro lens (Olympus Corporation, Tokyo, Japan). Image analysis was carried out for shoots and roots separately using Image J 1.36b (Wayne Rasband, NH, USA). Means of the pixel counts of the control seedlings were determined as 100% growth.

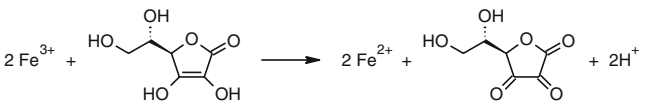
Postgermination Growth Assays (High Stress Scenario) Filter paper disks (Schleicher and Schuell 520 B 1/2, 60 mm diam) were moistened with 1 ml of a 10% methanolic aqueous solution containing the test compound in concentrations of 6–200 µg ml^{-1}. The moistened filter papers were placed in 9-cm Petri dishes. Surface-sterilized seeds were transferred to the filter paper with sterile forceps. Petri dishes were kept sealed for 48 h; then, the sealing tape was removed and the methanol was allowed to evaporate. Water was added to replenish the liquid and to keep the filter paper moistened. Scoring was performed as previously described. The assay was performed in dark at 25°C and scoring was performed after 5 days.

Statistics Statgraphics Plus 5.0 (Statistical Graphics Corp., Rockville, MD, USA) was used for statistics. Mann–Whitney rank test and analyses of variance (ANOVA) with Duncan's multiple range tests were performed at a confidence level of 95%.

Results and Discussion

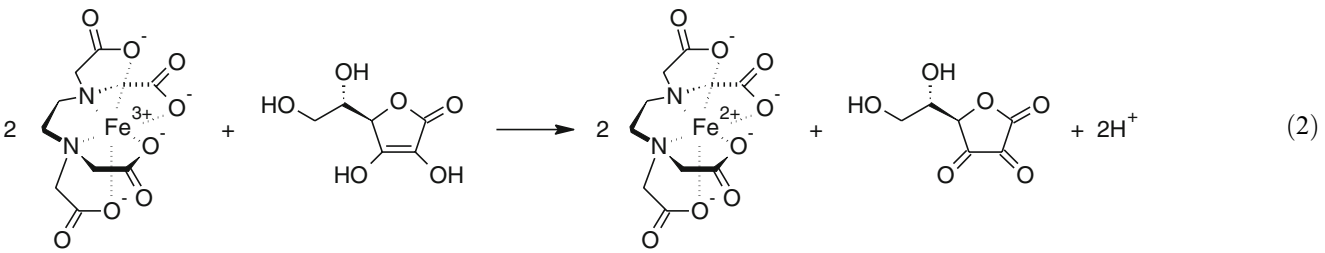
In the following text, we illustrate a number of reactions that aim to support our interpretation of the results obtained from the deoxyribose decomposition assays including one and two electron transfers. We stress that these reactions represent only a selection of possible ones, the majority of which probably are running simultaneously and reversibly. They are aimed at providing evidence that possible reactions exist that support the assay results.

Juglone proved to be a strong scavenger of hydroxyl radicals ($^{\bullet}OH$) in the deoxyribose assay (Fig. 1a). In this setup, ascorbic acid reduces iron (III) to iron (II). Ascorbic acid is oxidized to dehydroascorbic acid (1).



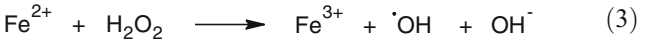
(1)

The same assay also was performed with the addition of EDTA, which efficiently chelates the iron ions but does not prevent iron from participating in further redox reactions. Consequently, ascorbic acid also reduced the EDTA-complexed iron (2).

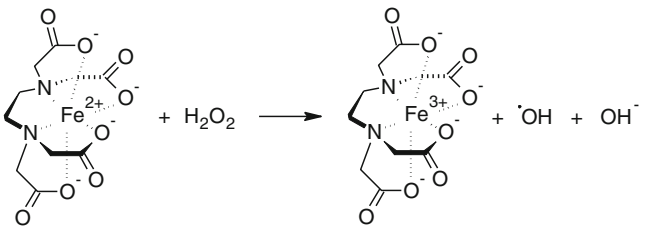


(2)

In our setup of the assay, the antioxidant activity was comparable to the variant without EDTA (Fig. 1a). This implies that chelation of iron by juglone does not affect its antioxidant activity. The reduced iron (Fe^{2+}), both in its free form (3) and as complex (4), reacts with hydrogen peroxide in the Fenton reaction and generates hydroxyl radicals, which are strong enough to decompose 2-deoxy-D-ribose to malonyldialdehyde (Aruoma et al. 1987; Aruoma 1994).

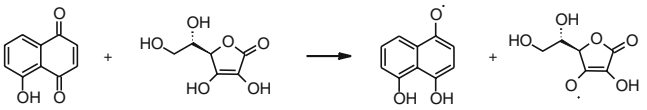


(3)

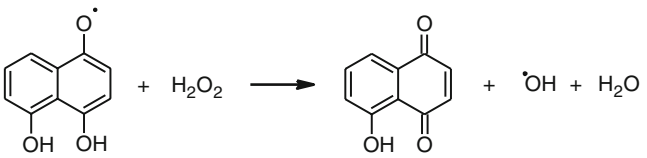


(4)

The hydroxyl radical also may arise by one electron transfers between the semiquinone of reduced juglone and hydrogen peroxide (5, 6).

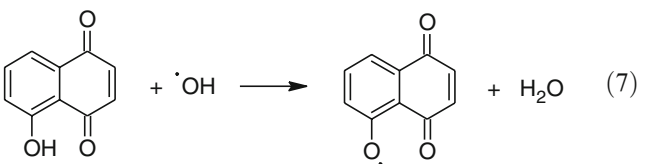


(5)



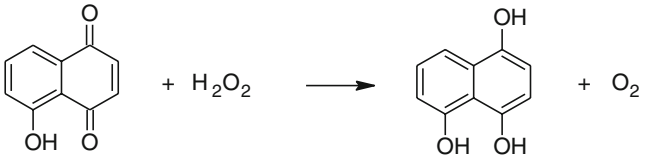
(6)

The fundamental reaction of the antioxidant activity of juglone is most likely the reduction of hydroxyl radicals to water (7).



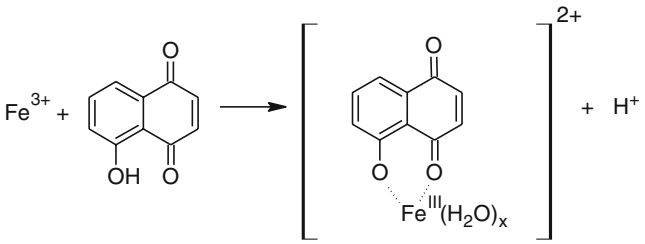
(7)

Alternatively, juglone may oxidize hydrogen peroxide to oxygen and thereby get reduced to trihydroxynaphthalene (8). The latter compound may be a more powerful reducing agent than juglone itself.



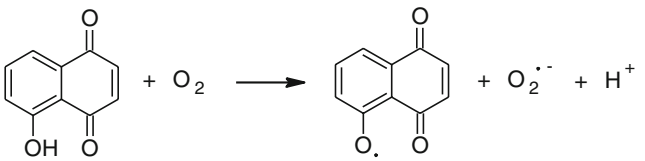
(8)

These reactions are possible at a more or less neutral pH (the pH of the aqueous buffer was adjusted to 7.4, thus resembling the milieu in the cytosol); at a lower pH, such as in the vacuole, they are less likely to work. In neutral milieu, juglone also may chelate iron ions (the chelation is both possible for iron (III) and iron (II) ions; the former are illustrated [9]). However, for antioxidant activity, chelation of iron ions by juglone did not affect the efficacy of the reaction.



(9)

In an attempt to obtain a tube assay that also facilitates the detection of prooxidative effects, the deoxyribose assay was modified. In this variant, hydrogen peroxide and ascorbic acid were not added. The duration of the assay was increased to 16 h to facilitate detection of reactions with oxygen dissolved in the aqueous buffer. Juglone showed a pronounced prooxidative effect as expected (Fig. 1b). Again, two setups were performed, one with addition of EDTA, the other without. However, the prooxidative activity of juglone was much more pronounced if EDTA was not added. This difference merits attention. Due to the absence of low concentration of free radicals, molecular oxygen is reduced to superoxide by juglone (10).



(10)

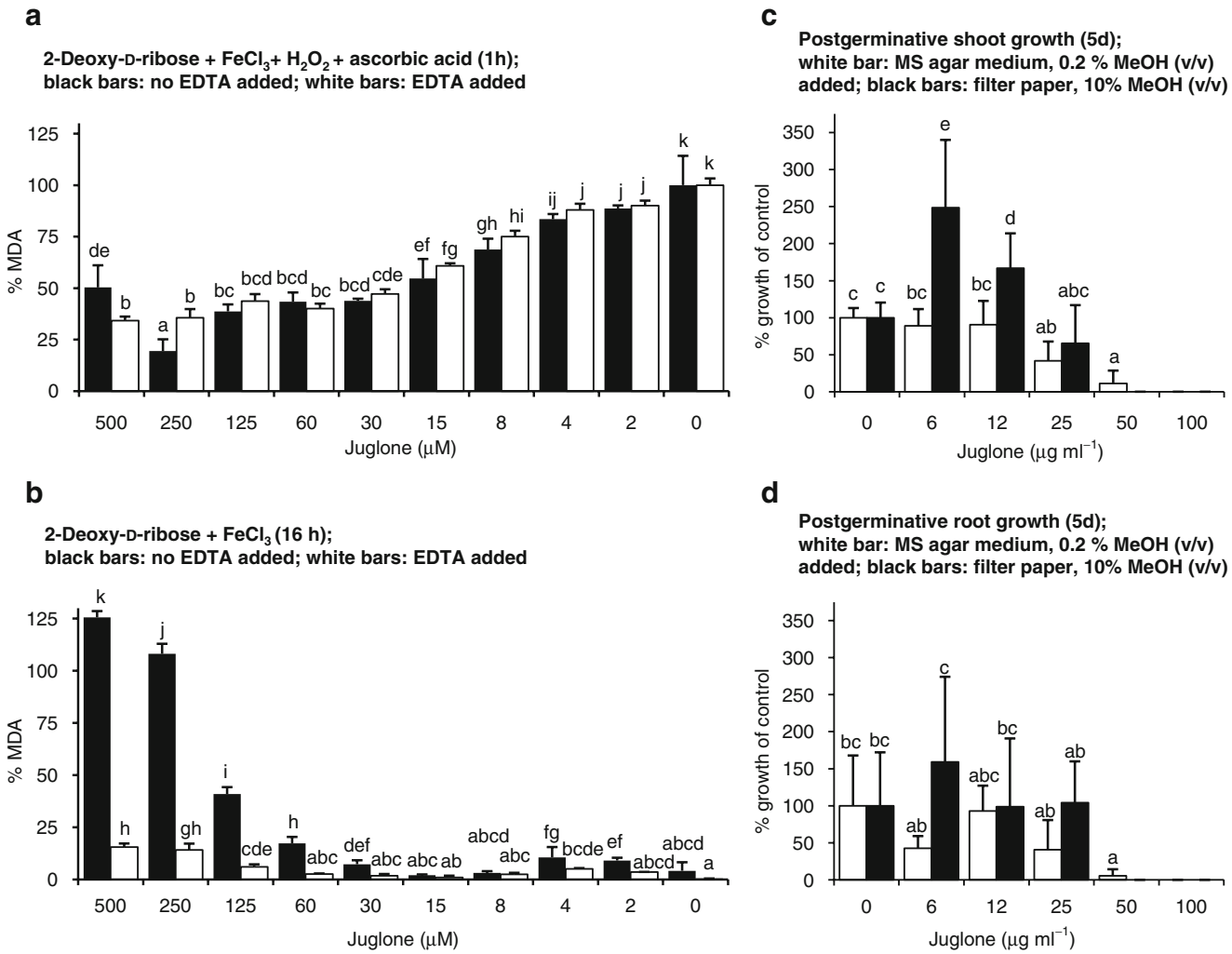


Fig. 1 **a** Antioxidative activity of juglone, quantified in % malonyldialdehyde, an oxidative decomposition product of 2-deoxy-D-ribose; *bars*, means; *error bars*, standard deviation; *N*=3, letters indicate different levels of significance (95% probability, Duncan's multiple range test). **b** Prooxidative activity of juglone; for specific information, see antioxidant activity; scale of 100% MDA scale is the same as in (**a**). **c** Postgerminative shoot growth, quantitative biological assay; *bars*, means; *error bars*, standard deviation; *N*=5; *letters* indicate different levels of significance (95% probability, Duncan's multiple range test). **d** Postgerminative root growth; for specific information, see shoot growth

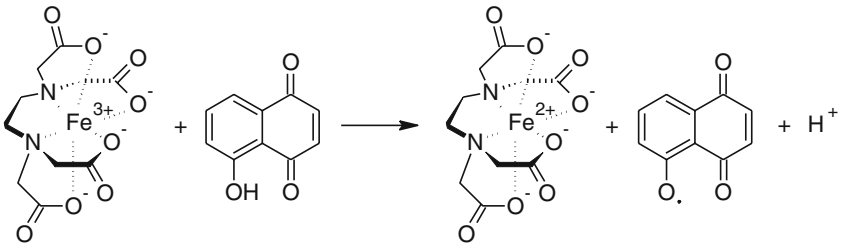
Superoxide can react with itself (dismutation of superoxide, [11]).

$$2\,O_2^{\bullet -} + 2\,H^+ \longrightarrow H_2O_2 + O_2 \qquad (11)$$

Superoxide may also directly react with the formed hydrogen peroxide generating hydroxyl radicals conditional to the presence of iron (12).

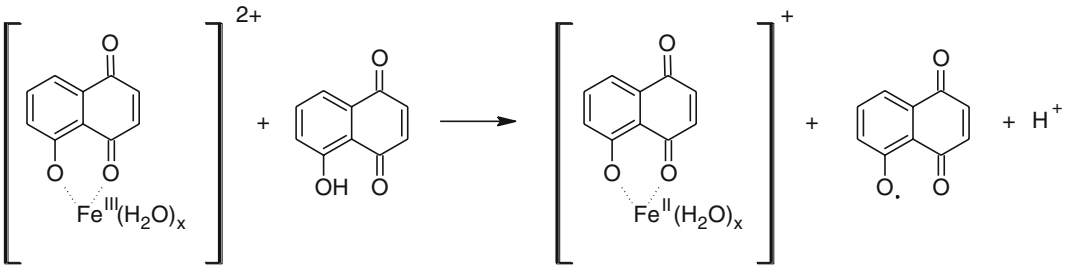
$$O_2^{\bullet -} + H_2O_2 \xrightarrow{Fe^{3+}} O_2 + {}^{\bullet}OH + OH^- \qquad (12)$$

The results suggest that chelation of iron by different molecules affects the electrochemical potential required for reduction of iron (III) (Miller et al. 1990). If EDTA is added, juglone reduces the EDTA complex of iron (III) (13).



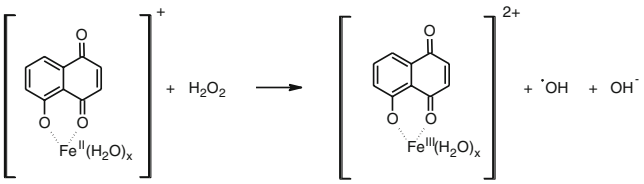
(13)

If no EDTA is added, then iron (III) is chelated by juglone and—as suggested by the pronounced activity visible at higher concentrations tested—reduced to iron (II) in the complex (14).



(14)

For the juglone–iron (III) complex, the required energy for the reduction seems to be considerably lower. As a consequence, more juglone-chelated iron (II) is available for the generation of hydroxyl radicals in a Fenton type reaction (15) than EDTA-chelated iron (II) in the other setup.



(15)

Within the tested range of concentrations, the prooxidative effect was linear. It is possible that the nonlinearity in the antioxidant setup, where no EDTA was added, may be caused by the reactions leading to the prooxidative effect as described above.

By starting from the notion that juglone is an efficient redox cycler, we wanted to explore if juglone might cause nonlinear effects in biological assays, hormesis—a process whereby test organisms exposed to low levels of stress or toxins become relatively more resistant to subsequent challenges. In the present case, we expected low levels of juglone to ameliorate the stress caused by higher methanol concentrations. In vivo oxidation of methanol may generate superoxide, thus causing toxic effects in living organisms. The addition of methanol to aqueous solutions may not only be employed to improve dispersion of lipophilic compounds in aqueous media—as is good laboratory practice—but also to increase stress for the tested organism. Thus, to create a low- and high-stress setup of the postgermination growth assay, *S. alba* was germinated on MS agar medium (<0.2% MeOH) and on filter paper assay (10% MeOH). The results are illustrated by Fig. 1c and d. Shoot size of the seedlings exposed to higher methanol concentration was nearly half the size of seedlings exposed to the low methanol concentration (P=0.005, Mann–Whitney rank test). Figure 1c and d illustrate the postgerminative growth dynamics as % in relation to the control; thus, the inhibitory effect of the higher methanol concentration is not evident, as both means of the control denote 100%.

Root development was not significantly affected by the various methanol concentrations (P=0.689, Mann–Whitney rank test). At lower methanol concentrations (<0.2%), increasing concentrations of juglone inhibited postgermination growth of *S. alba* seedlings clearly affecting the shoot and less evidently the root; 100 μg ml^{-1} completely inhibited germination. The concentration of the solution of juglone in the agar was about 575 μM. At the same concentration range, the deoxyribose degradation assay suggests that juglone may cause a pronounced prooxidative effect when levels of free radicals are low (Fig. 1b). In the filter paper assay (the initial concentration of methanol was around 10%), germination was inhibited at 50 μg ml^{-1} (287 μM). Although, at 6 μg ml^{-1} (34 μM), a significant stimulatory effect was visible (Fig. 1c). This stimulation of lower juglone concentrations was especially visible in the shoot development, which similarly was more affected by higher methanol concentrations. The roots were less affected and, accordingly, were less stimulated by lower juglone concentrations. At the low concentrations where the stimulatory and thus hormetic effect was visible (±30 μM), the deoxyribose degradation assay suggests that juglone may efficiently scavenge present hydroxyl radicals. This also reflects a mitigating effect on the stress when the seeds were exposed to high methanol concentrations.

Cypser and Johnson (2002) observed a similar hormetic effect of juglone in gerontological studies that used the nematode *C. elegans* as a test organism. In that study, pretreatment with the toxin juglone mitigated oxygen stress. Conversely, preceding oxygen stress also mitigated stress caused by the application of juglone. These authors concluded that low levels of initial oxidative stress

obviously mitigate effects of subsequent stressors that cause oxidative stress. Here, we demonstrated that juglone can scavenge hydroxyl radicals generated by Fenton type reactions during oxidative stress in a chemical assay. In our biological assay, a hormetic effect was visible only when seeds of *S. alba* were stressed with 10% methanol in the solution. The results suggest that the hormetic effect of a redox cycling compound (in this regard, juglone represents an efficient example) may be caused by scavenging ROS such as the hydroxyl radical.

Juglone is a potent bioactive compound. Can this compound, however, serve as an effective chemical defense, for example, in the case of allelopathy of the walnut tree? This question is difficult to answer, as it is difficult to develop one ideal assay to measure pro- and antioxidative activities (Halliwell and Gutteridge 1995). Hormetic activity may be determined not only by structural characteristics and concentration, but also—as our results suggest—be facilitated by the milieu in the tissues of the targeted organism. The results presented here provide ideas for explaining hormesis caused by juglone. Evolution may favor such potent redox cyclers. Gene expression studies in *Arabidopsis thaliana* and rice have shown that juglone (Mylona et al. 2007), herbicides such as paraquat and glyphosate (Ahsan et al. 2008), and even hormones such as abscisic acid (Guan et al. 2000), may by generating ROS provoke similar effects in the expression patterns of antioxidant genes. This suggests that common stress-related motifs in the promoters of these genes should be present (Mylona et al. 2007). Studies that assess the effect of allelochemicals on cell cycling (Sanchez-Moreiras et al. 2008) should increase our understanding of allelopathy.

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Ontogenetic Variation in the Chemical Defenses of Cane Toads (*Bufo marinus*): Toxin Profiles and Effects on Predators

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Abstract We conducted a quantitative and qualitative chemical analysis of cane toad bufadienolides—the cardioactive steroids that are believed to be the principal cane toad toxins. We found complex shifts in toxin composition through toad ontogeny: (1) eggs contain at least 28 dominant bufadienolides, 17 of which are not detected in any other ontogenetic stage; (2) tadpoles present a simpler chemical profile with two to eight dominant bufadienolides; and (3) toxin diversity decreases during tadpole life but increases again after metamorphosis (larger metamorph/juvenile toads display five major bufadienolides). Total bufadienolide concentrations are highest in eggs (2.64±0.56 μmol/mg), decreasing during tadpole life stages (0.084±0.060 μmol/mg) before rising again after metamorphosis (2.35±0.45 μmol/mg). These variations in total bufadienolide levels correlate with toxicity to Australian frog species. For example, consumption of cane toad eggs killed tadpoles of two Australian frog species (*Limnodynastes convexiusculus* and *Litoria rothii*), whereas no tadpoles died after consuming late-stage cane toad tadpoles or small metamorphs. The high toxicity of toad eggs reflects components in the egg itself, not the surrounding jelly coat. Our results suggest a dramatic ontogenetic shift in the danger that toads pose to native predators, reflecting rapid changes in the types and amounts of toxins during toad development.

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Introduction

Understanding the pathways by which introduced species modify the ecosystems they invade is a central challenge for conservation biology; unless we understand those effects, we are unlikely to mitigate them. However, the challenge is a daunting one because of the diverse array of mechanisms involved in the ecological impact of invasive species. Some introduced species act as predators, some as competitors, and others as parasites (Sandlund et al. 1999; Mack et al. 2000; Mooney and Cleland 2001; Schlaepfer et al. 2002). Another category of impact involves toxic invaders, species that imperil native species via exposure to toxins lethal to the native taxa. If an invading species belongs to a phylogenetic lineage not represented in the fauna of the invaded area, it may possess toxins to which native predators are naïve and, hence, are unable to tolerate. For example, Australia has no endemic bufonids ("toads"), and the invasion of South American cane toads (*Bufo marinus*; see Frost et al. 2006; Pramuk 2006; Pramuk et al. 2008 for alternative generic allocations) has killed a diverse array of Australian predators (including crocodiles, marsupials, snakes, lizards, and anurans) through poisoning when they attempt to eat the toxic invader (Burnett 1997; Doody et al. 2006; Griffiths and McKay 2007; Crossland et al. 2008; Letnic et al. 2008).

Although conventional wisdom (including publications for the general public from government departments and museums) asserts that all life history stages of cane toads are highly toxic to predators, this generalization is not supported by empirical evidence. Although it is more than a

century since Phisalix (1903, 1922) reported that toxicity of bufonid larvae (*Bufo bufo*) decreased with increasing tadpole stage, few empirical data are available on the toxicity of bufadienolides from cane toads (Lever 2001). This lack of empirical support for the conventional wisdom motivated the present study.

To our knowledge, there have been no previous analyses of the chemical composition of bufonid toxins as a function of ontogeny. The most direct evidence of shifts in toxicity comes from studying the effects of toad eggs and larvae on predators. Crossland and Alford (1998) reported no apparent ontogenetic change in toxicity of cane toads (for example, both eggs and hatchlings of toads were toxic to frog tadpoles but not to crayfish), but their experiments were not designed to focus specifically on ontogenetic shifts. In a more detailed study, Crossland (1998) reported that belostomatid water bugs experienced higher mortality rates from consuming late-stage toad tadpoles than from eating earlier-stage conspecifics. Ontogenetic changes in palatability of toad tadpoles also hint at underlying toxicity (presumably, predators detect and avoid toxins). Several authors have reported that the palatability of toad tadpoles differs among developmental stages (see review by Gunzburger and Travis 2005). For example, Heyer et al. (1975) personally tasted small and large tadpoles of *B. marinus* and reported that larger tadpoles were less palatable. Similarly, Lawler and Hero (1997) found that palatability of cane toad tadpoles to fish (barramundi, *Lates calcarifer*) decreased during ontogeny.

In adult toads, toxins are localized largely in parotid glands, where they can be expressed as a secretion during a predatory event. At the molecular level, the toxic chemicals in cane toad parotid secretions are attributed to steroids known as bufadienolides (Lever 2001). Bufadienolides are not unique to toads and are known to exert a cardiotoxic effect by inhibiting membrane-bound heart tissue Na^+/K^+ adenosine triphosphatase (ATPase; Steyn and van Heerden 1998). The potency and selectivity of toxicity vary among different bufadienolides and with different isoforms of Na^+/K^+ ATPase in different tissues and animal species (e.g., Akimova et al. 2005; Keenan et al. 2005). Given this variability in sensitivity to cane toad bufadienolides, it is impossible to establish a single definitive bioassay—biochemical-, cell-, or animal-based—that measures absolute toxicity. To generate ecologically relevant measures of "overall toxicity," we need detailed analysis not only of the toxin components within each ontogenetic stage but also of their relative toxicity to predators. The latter measures could be obtained in a standard Na^+/K^+ ATPase bioassay, but this is not definitive. A more ecologically relevant assay would be based on actual measures on the predator species of concern (in the case of cane toads in Australia, this would involve taxa such as varanid lizards, crocodiles, and quolls), but this becomes prohibitive (on both logistical and ethical grounds) when each species requires its own optimized assay. Rather than this, we used a chemical approach to assess likely levels of toxicity by measuring the presence and composition of the different bufadienolides. Australian cane toad parotid secretions are dominated by several major bufadienolides (some of which have not been reported previously from parotid secretions), with >90 additional, as yet unidentified, bufadienolides occurring at low concentrations (Hayes and Capon, unpublished). Although many of these minor cane toad bufadienolides are present at concentrations <0.1% of that of the major bufadienolides, bufadienolide potency against Na^+/K^+ ATPase can span several orders of magnitude (e.g., Wallick and Schwartz 1988).

These observations highlight the importance of establishing sensitive analytical methods capable of detecting and quantifying the full complement of cane toad bufadienolides, whether from secretions or whole animals and across all life history stages. The relative toxicity of cane toad eggs, tadpoles, and metamorphs bears directly on the ecological impact of this invasive species. For example, the toxicity of eggs and tadpoles determines the risk of poisoning to aquatic fauna (e.g., fishes, native tadpoles), whereas the toxicity of metamorph toads determines the risks faced by predators that forage for small prey at the margins of breeding ponds (such as adult frogs, small snakes, and waterbirds). Because alternative toxins are likely to be differentially toxic to different types of predators (e.g., vertebrates vs. invertebrates: Crossland and Alford 1998), we also need to document toxin identities. Hence, understanding the distribution of toxin types and contents during toad ontogeny can provide a useful background for interpreting the ecological impact of this invasive anuran. To clarify these issues, we measured toxin content of eggs, larvae, and postmetamorphic toads, and we also monitored mortality of predators exposed to these life history stages.

Methods and Materials

Collection, Preparation, and Chemical Analysis of Samples We obtained four clutches of eggs from captive toads collected in the Townsville region of Queensland (two pairs) and the Adelaide River floodplain of the Northern Territory (two pairs). Spawning was induced by subcutaneous injections of 0.5–0.75 ml of leuprorelin acetate (Lucrin, Abbott Australasia) diluted in amphibian ringers' solution to a concentration of 0.25 mg/ml. Soon after oviposition, we collected fertilized eggs, some of which were frozen and stored at −80°C. After hatching, tadpoles were kept in water conditioned with aquaria water ager at a

pH of 7.5. We fed tadpoles with boiled lettuce *ad libitum* (twice a week) and changed the water weekly.

A subset of tadpoles were euthanized at Gosner (1960) development stages 21, 25, and 44/45 and then stored frozen at −80°C. Tadpoles from each of the four clutches were left to complete metamorphosis, at which time some were frozen (snout–urostyle length [=SUL] 6.0–9.0 mm, mean=8.0 mm; mass=20.2–90.0 mg, mean=48.9 mg), and the remainder were kept separately by clutch in 54-l containers (45×40×30 cm) on a substrate of moist sand and water, with shelter items for refuge. The enclosures were illuminated by fluorescent tubes (photoperiod 12:12 h L/D cycle), with 75% relative humidity, and ambient temperature at 32°C. The toads were fed live crickets *ad libitum*. We cleaned the enclosures weekly and changed the water daily. After 60 days postmetamorphosis, toad metamorphs/juveniles (SUL 14.0–34.0 mm, mean=24.2 mm; mass 0.51–3.59 g, mean=1.73 g) from each of the four clutches were euthanized and stored frozen at −80°C.

To identify and quantify specific bufadienolides, tissue samples from each life history stage from each clutch (N=8) were homogenized (IKA T10 Basic Ultra Turrax with S10N-5G dispersion tool) in water (1 ml). Each homogenate was made up to equal volumes of water and *n*-butanol, and phases were left to separate at 4°C overnight. Samples were filtered with filter aid (Celite), divided into water and *n*-butanol fractions, and evaporated to dryness *in vacuo* at 40°C. The mass of each fraction was determined, and *n*-butanol fractions were diluted to a final concentration of 2 mg/ml with methanol. High-performance liquid chromatography (HPLC) was performed with an Agilent 1100 Series Separations Module equipped with an Agilent 1100 Series diode array detector (DAD) and running ChemStation Rev. B0201SR1 software. We performed electrospray ionization mass spectrometry (MS) using an Agilent 1100 Series Separations Module equipped with an Agilent 1100 Series liquid chromatograph/mass selective detector. HPLC gradient conditions were as follows: 1-ml/min gradient elution from 90% H_2O/MeCN (0.01% trifluoroacetic acid, TFA) to 50% H_2O/MeCN (0.01% TFA) over 20 min, then to MeCN (0.01% TFA) over 5 min, followed by a 5-min flush with MeCN. The HPLC-DAD-MS gradient was identical to above except for the use of 0.05% HCOOH as a modifier in the place of TFA, in order to minimize ion suppression in the negative mode. The column used for both gradients was a Phenomenex Onyx Monolithic C_{18} 100×4.6-mm column.

We inferred the presence of bufadienolides by examination of the UV-Vis spectrum of each peak, with the α-pyrone ring having a distinctive absorption maximum at 297 nm. For those components where the molecular weight could be ascertained, we assigned tentative identity to several of the detected components, based on this molecular weight, the characteristic UV-Vis spectra, comparison of retention time to bufadienolides from adult cane toad parotid secretion [purified and characterized by HPLC-DAD and ^{1}H and ^{13}C nuclear magnetic resonance (Hayes and Capon, unpublished)], and previous reports from this and related toad species (Akizawa et al. 1994; Matsukawa et al. 1994; Steyn and van Heerden 1998).

Bioassay of Toad Palatability and Toxicity We obtained eggs from cane toads collected on the Adelaide River floodplain of the Northern Territory and raised them through to metamorphosis in large outdoor enclosures. To quantify toxicity of eggs, tadpoles, and metamorphs to native predators, we needed a predator that might encounter and consume all stages in the toad's early life history (eggs, larvae, and metamorphs). Tadpoles of native frogs were chosen as predators because field studies show that these animals frequently consume toad eggs and often die as a result (Crossland et al. 2008). Although frog tadpoles rarely kill live toad tadpoles or metamorphs, they readily consume dead tadpoles and metamorphs (Crossland and Alford 1998; Crossland and Azevedo-Ramos 1999; M. Crossland personal observation). Egg clutches of two frog species (*Limnodynastes convexiusculus* and *Litoria rothii*) were collected from the Adelaide River floodplain and raised in large outdoor enclosures. To quantify the level of risk posed by toads at different life history stages, we placed ten eggs, a dead tadpole, or a dead metamorph toad together with a native tadpole (*L. convexiusculus* Gosner stages 25 to 37; snout–vent length [=SVL] 7.21–20.70 mm; *L. rothii* Gosner stages 27 to 38; SVL 9.36–27.32 mm) in a 1-l plastic container. We used toad tadpoles at three developmental stages: early stage (Gosner stages 25 to 28; SVL 4.7–6.2 mm, mean=5.0 mm), midstage (Gosner stages 30 to 35; SVL 7.7–9.0 mm, mean=8.5 mm), and late stage (Gosner stages 40 to 42; SVL 13.2–14.6, mean=13.9 mm). Two size classes of metamorph toads were tested: (1) 10.9–12.9 mm SUL (mean=12.1 mm) and 0.11–0.18 g (mean=0.15 g) and (2) 16.0–19.4 mm SUL (mean=17.4 mm) and 0.38–0.77 g (mean=0.48 g). Depending on availability, we ran either 20 or 40 native tadpoles as experimental animals plus the same number of controls (run concurrently, kept individually in identical containers, but without the toad stimulus). After 24 h, we quantified consumption of toads and scored whether the native tadpole was alive or dead and, if alive, whether it was overtly affected by exposure to toad material (impaired swimming ability).

As in other anuran species, eggs of cane toads consist of an ovum surrounded by a jelly coat. The jelly coat consists of several layers, each of which may have specific biological functions (e.g., Duellman and Trueb 1986). Thus, the rapid decline in toxicity from toad eggs to toad

tadpoles (see below) might reflect, at least partly, high concentrations of toxin in the jelly coat (Gunzburger and Travis 2005). To test this hypothesis, we carefully separated eggs from their jelly coats and exposed tadpoles of three frog species (N=20 per treatment for *Litoria caerulea*, *Litoria nasuta*; N=10 per treatment for *Opisthodon ornatus*) to the jelly coat material, plus appropriate controls (ten intact toad eggs per container; and no egg material per container). All tadpoles were in individual containers, as before.

Results

Toxin Profiles Figure 1 compares chromatograms of the *n*-BuOH extracts of each ontogenetic stage and reveals differences among toxin profiles of different ontogenetic stages of the cane toad. Six of the bufadienolides found in these stages were characterized by HPLC-DAD-MS, and their percentage occurrence in each stage is shown (Table 1). The unique UV spectrum of the bufadienolides allowed their assignment to this class of compound, even if a complete chemical identity was not obtainable, and in this case the number of additional bufadienolides is given (Table 1). The number of bufadienolide peaks found uniquely in each ontogenetic stage is also given (Table 1).

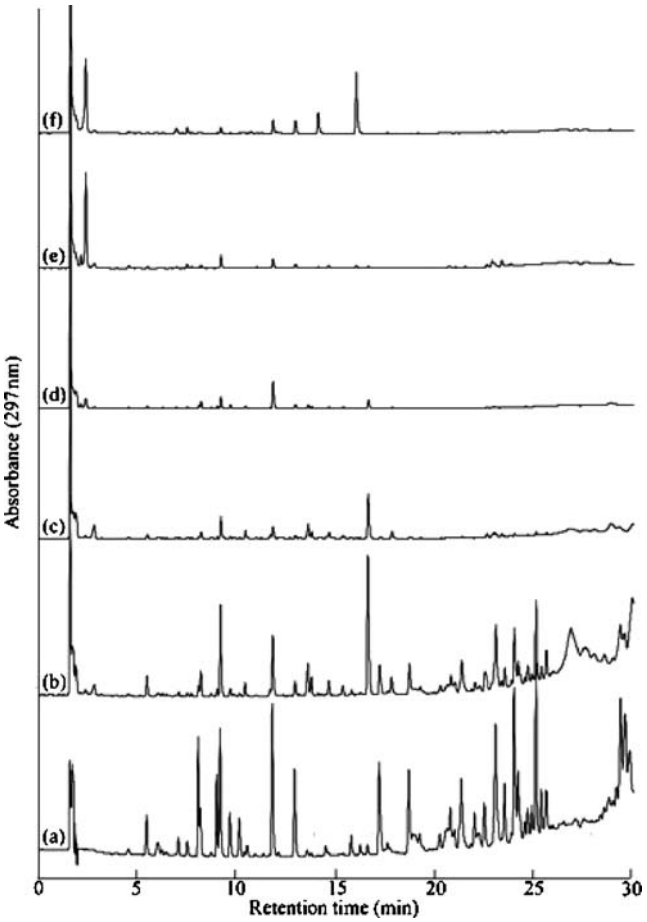

Fig. 1 Stack plot of sample HPLC chromatograms of *n*-BuOH extract from each studied ontogenetic stage of cane toads, analyzed at 297 nm. Stage of each plot is indicated: (*a*) eggs, (*b*) stage 21, (*c*) stage 25, (*d*) stage 44/45, (*e*) small metamorph, (*f*) large metamorph/juvenile. Gradient: 100% H_2O for 5 min, gradient to 25% MeOH over 5 min, isocratic for 10 min, then gradient to 100% MeOH over 5 min, using Phenomenex Onyx Monolithic C_{18} column (100×4.6 mm)

Analyses across different ontogenetic stages showed that the total number of bufadienolide peaks varied with stage ($F_{5,\ 48}$=25.16, P<0.001; Fig. 2a), as did the mean amount of bufadienolide (in micromole per milligram of extract; $F_{5,\ 48}$=5.462, P<0.001; Fig. 2b). Multiple comparisons determined with Tukey test are displayed in Fig. 2. In each case, the eggs had the highest values, both in number of peaks and in amount of bufadienolide. In the case of peak numbers, there was no significant difference between the number of bufadienolide peaks in all tadpole and early postmetamorphic stages, except for another significant drop at stage 25 (Fig. 2a). Stage 25 was the only stage to have a significantly reduced amount of bufadienolide (Fig. 2b), although a trend is apparent whereby eggs and larger metamorphs/juveniles had the most bufadienolide, with a reduction during the larval stage and immediately post-metamorphosis.

Bioassay of Toad Palatability and Toxicity In both predator species that we tested, palatability and toxicity of cane toads decreased dramatically through ontogeny (Figs. 3 and 4). Controls did not exhibit any mortality or abnormal behavior over the course of trials, in any experiment. Palatability of toads declined gradually through early toad ontogeny for *L. convexiusculus* tadpoles but remained high before an abrupt decline in *L. rothii* tadpoles (Fig. 3). Consumption of toad eggs was invariably fatal to frog tadpoles in our trials (one to ten eggs eaten), whereas feeding on late-stage larvae or metamorphs (including complete consumption) did not kill any test animals. The proportion of tadpoles adversely affected by toad consumption declined rapidly; early-stage toad tadpoles posed a significant danger whereas late-stage toad tadpoles and smaller metamorphs did not (Fig. 4). Sublethal toxic effects did not become apparent again until tadpoles fed on larger metamorphs (*L. convexiusculus* only; Fig. 4). The lack of mortality after exposure to larger metamorphs (Fig. 4) presumably reflects their low palatability to both tadpole species (especially *L. rothii*: Fig. 3) and, thus, says little about their actual toxicity. Contingency table tests show that the ontogenetic stage of the toad strongly influenced the probability that a predator would survive toad consumption, in both species of native frog (*L. convexiusculus*, χ^2=76.96, df=3, P<0.001; *L. rothii*, χ^2=99.07, df=5, P<0.001). Similarly, the probability of surviving after encoun-

Table 1 Percentage occurrence and tentative identity of bufadienolides from each life history stage of cane toads examined

Tentative identity	Percent occurrence by ontogenetic stage					
	Eggs	Stage 21	Stage 25	Stage 44/45	Meta	Juv
Arenobufagin	75	75	25	89	100	100
Hellebrigenin	75	63	–	33	50	–
Telocinobufagin	75	75	–	89	100	100
Marinobufagin	75	50	–	33	88	100
Bufalin	–	–	–	–	–	100
Resibufagenin	38	–	38	89	–	100
Number of additional bufadienolides	23	7	1	8	7	–
Number of unique bufadienolides	17	1	–	–	1	1

The final two rows show the number of additional unidentified bufadienolides from each stage and the number of unique bufadienolides found in each stage that were not detected in any other stage. "–" means below detectable levels. The "Meta" column refers to small metamorphs (<13 mm SUL) and the "Juv" column refers to a mixture of larger metamorphs and juveniles (i.e., >16 mm SUL). Mean amounts of each component shown in Table S1 in Supplementary Data

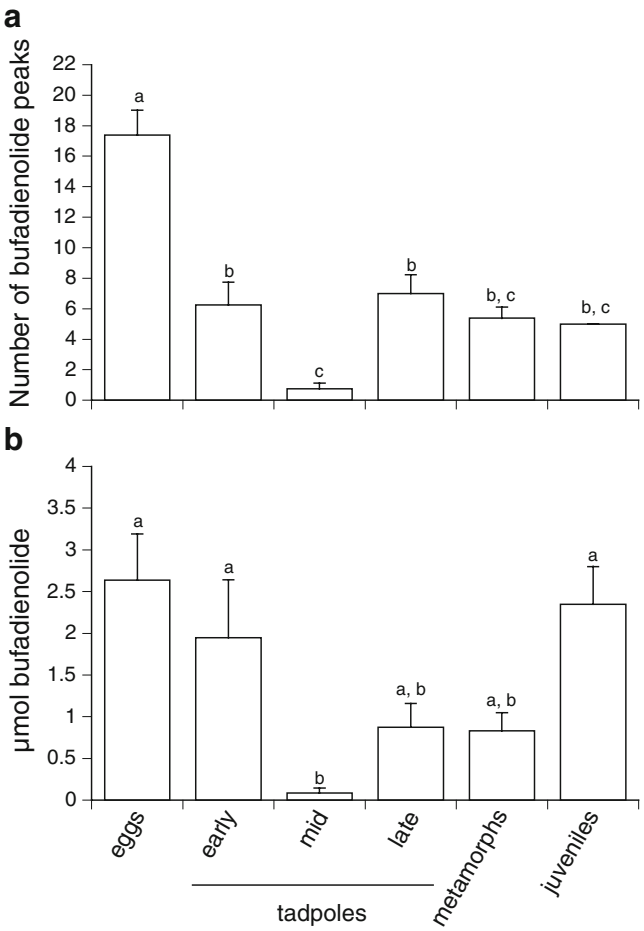

Fig. 2 Changes in the bufadienolide content and diversity across ontogenetic stage: **a** mean number of bufadienolide peaks detected and **b** total amount of bufadienolide (μmol). All data shown as mean± SEM. The "metamorph" category refers to small metamorphs (<13 mm SUL), whereas the "juveniles" category refers to metamorphs >16 mm SUL. Differing *small letters above bars* indicate statistical significance at $P<0.001$

tering (but not necessarily eating) toads differed strongly among toad age classes (*L. convexiusculus*, $\chi^2=108.35$, $df=5$, $P<0.001$; *L. rothii*, $\chi^2=116.42$, $df=5$, $P<0.001$).

Trials to identify the location of the toxin (ovum vs. jelly coat) also yielded clear patterns. All tadpoles placed with jelly coat (or controls with no egg materials) survived and exhibited normal behavior. All tadpoles in the jelly coat trials consumed the jelly that was offered. Intact eggs posed a greater danger. For *L. caerulea*, 13 of 20 tadpoles placed with intact eggs died, after consuming 0.5 to two embryos. Another three tadpoles ate one to two embryos and survived; the remainder did not eat during the trials. The *L. nasuta* and *O. ornatus* tadpoles placed with toad eggs consumed 0.5 to six eggs each, and all died.

Discussion

Both the total amount of toxin and its composition (relative proportions of different bufadienolides) change considerably through the course of toad ontogeny (Fig. 1, Table 1). Notably, newly laid toad eggs contained high levels of bufadienolides, whereas midstage tadpoles exhibited lower amounts, and all stages after the egg show a decreased diversity of toxins (Fig. 2). In the absence of detailed information on the toxicity of each type of bufadienolide to each type of potential predator, we cannot translate data on bufadienolide content directly into a measure of the danger posed to a predator.

Nevertheless, our data on toxicity of early-stage toads to native predators (frog tadpoles) fit well with the results of our chemical analyses, especially regarding diversity of the toxins. Toad eggs were invariably deadly to frog tadpoles, but the risk of poisoning fell rapidly through toad larval

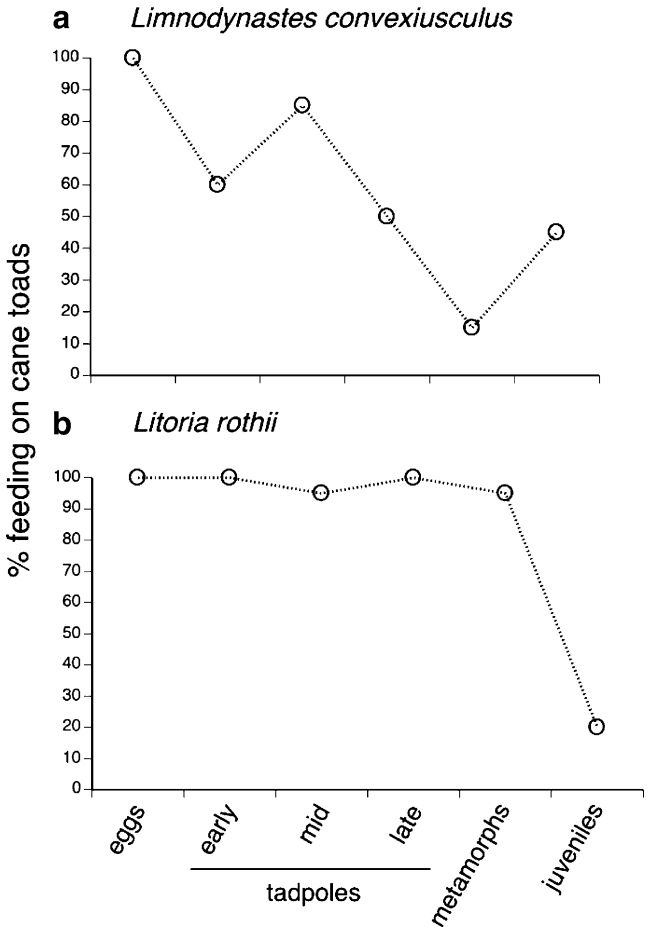

Fig. 3 Ontogenetic shifts in the palatability of early life history stages of cane toads (*B. marinus*) to tadpoles of two Australian frog species, *L. convexiusculus* and *L. rothii*. Captive tadpoles were exposed to eggs, tadpoles, and metamorphs of cane toads. We define palatability based on the proportion of predators that attempted to consume toad tadpoles. The "metamorph" category refers to small metamorphs (<13 mm SUL), whereas the "juveniles" category refers to metamorphs >16 mm SUL

life, was minimal for frog tadpoles encountering late-stage toad tadpoles or small toad metamorphs, and became evident again when feeding on larger metamorphs (Fig. 4). A similar general pattern of unpalatability declining from earlier to later stages during anuran early ontogeny seems to be widespread in amphibians (Gunzburger and Travis 2005) including bufonids (Brodie et al. 1978; Brodie and Formanowicz 1987). Thus, although intuition suggests that larger toad tadpoles should contain more toxins (Heyer et al. 1975) and some previous experiments have shown exactly that shift in either palatability (Lawler and Hero 1997) or toxicity (Crossland 1998), our study shows a decreasing toxicity through development for toads consumed by frog tadpoles.

The chemical analyses similarly reveal large and rapid ontogenetic shifts in toxin content (both concentration and diversity) during the cane toad's early life history. High concentrations of bufadienolides in the ovary of reproducing female toads and high toxicity of toad eggs have been reported by several authorities (e.g., Licht 1968; Lever 2001). Experiments in which we separated the egg into their two main components localize the toxin clearly to the ovum, not the jelly coat. The jelly coat may have multiple biological functions including facilitation of fertilization and protection against physical injury, fungal infection, thermoregulation, and embryo hydration (see review by Duellman and Trueb 1986). Larger firmer egg capsules deterred predation by fishes (Grubb 1972); and removal of the egg capsule or jelly coat increased vulnerability to predation (Licht 1968; Werschkul and Christensen 1977; Jennings and Schaefer 1978; Ward and Sexton 1981). However, these studies could not assess whether protection

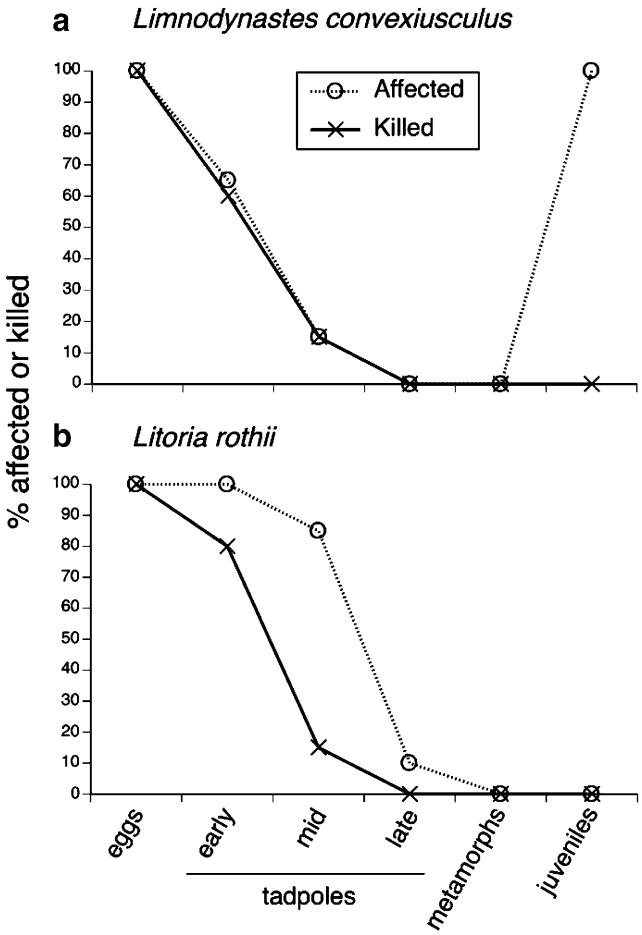

Fig. 4 Ontogenetic shifts in the toxicity of early life history stages of cane toads (*B. marinus*) to tadpoles of two Australian frog species, *L. convexiusculus* and *L. rothii*. Captive tadpoles were exposed to eggs, tadpoles, and metamorphs of cane toads. Control tadpoles all survived, so data for these groups are not shown. The "metamorph" category refers to small metamorphs (<13 mm SUL), whereas the "juveniles" category refers to metamorphs >16 mm SUL

was gained from chemical or physical properties of the jelly coat, and we are not aware of any experimental demonstration that identifies the location of toxins in amphibian eggs (albeit, we note that Phisalix [1922] concluded that "the toxic components of *Bufo* eggs appear to be in the ovarian egg, and the jelly coat gives added protection"). Our results implicate the ovum and not the jelly coat as the source of toxins in cane toads.

Ontogenetic shifts in toxicity may have major ecological consequences (Hanifin et al. 2004); for example, the danger posed by cane toads to native predators shifts dramatically through toad ontogeny. In the case of the frog species that we studied, the risk level changed from almost certainly fatal (ingestion of eggs) to minimal risk (ingestion of late-stage larvae and small metamorphs), before increasing again for larger metamorphs. The broad conservatism in physiological determinants of vulnerability to bufadienolides among vertebrates suggests that other vertebrate predators will exhibit similar patterns of vulnerability. For the minority of predator species with high tolerance to these toxins, no life history stage of the cane toad will be threatening (Peterson and Blaustein 1992; Lever 2001). In a country like Australia, however, which lacks native bufonids, many predators have low resistance to the cane toad's toxins (Lever 2001; Phillips et al. 2003). For most of this latter group, we predict that toad eggs will be more toxic than either tadpoles or small metamorphs. The stage of postmetamorphic life when the young toads begin to produce their own toxins may vary among *Bufo* species: for example, Brodie et al. (1978) reported high levels of unpalatability for *Bufo americanus* within 24 h after metamorphosis.

Patterns of vulnerability may also differ for other types of predators. The physiological pathways by which arthropods are affected by bufadienolides differ considerably from those of vertebrates. Insect Na^+/K^+ ATPase lacks the binding site for cardenolides (Okimura et al. 2002) and thus presumably the site for binding of bufadienolides, and these toxins will not have the same effect on insect predators. A recent review of literature concluded that, overall, insect predators are less likely to find amphibian eggs unpalatable than are vertebrate predators (Gunzburger and Travis 2005). Many invertebrates are unaffected by cane toad toxins (e.g., crustaceans, odonates, nepids, dytiscids), but others are vulnerable (e.g., snails—Crossland and Alford 1998). Intriguingly, Crossland (1998) reported that late-stage cane toad tadpoles were more toxic to belostomatid water bugs than were early-stage tadpoles: the reverse of the pattern was documented in the present study. This toxicity is presumably related to some other unidentified toxin or toxins rather than to bufadienolides (that are decreased in diversity at this stage). Further work to test this hypothesis would be of interest, and interspecific variation may be considerable. For example, newly hatched toad (*B. americanus*) larvae are less palatable to invertebrate predators than midstage or late-stage tadpoles (Brodie et al. 1978; Brodie and Formanowicz 1987).

Given the invariably fatal outcomes from ingesting toad eggs (Fig. 4), why do frog tadpoles attack these dangerous meals? This situation may reflect the lack of evolutionary contact between frog species in our Australian study area with the invasive cane toad (that arrived only 2 years before the experiments were conducted). Even in species that have evolved with toads, selection to avoid eggs may be weak if the eggs typically are laid in places and at times where they would rarely be encountered by frog tadpoles. In Gunzburger and Travis' (2005) review, *Bufo* eggs, hatchlings, and tadpoles were rejected as prey in only 20% of trials—most of which dealt with predators sympatric with bufonids over evolutionary timescales. None of the studies reviewed by Gunzburger and Travis reported avoidance of bufonid eggs by heterospecific tadpoles.

Further, what do our results tell us about the ecological impact of cane toads in Australia and the kinds of methods that we might use to mitigate that impact? The degree of risk to a predator will depend upon the toxin content in toads (which may vary geographically and among individuals: Daly et al. 2008) as well as upon the life history stage of cane toads that the predator attempts to consume. As is well known, adult cane toads are highly toxic (our analysis of adult toad parotid glands reveals five dominant and ~100 minor bufadienolides: R. A. Hayes and R. J. Capon unpublished data), but the situation is more complex with earlier life history stages. A vertebrate predator will be at greater risk if it attempts to consume toad eggs rather than toad tadpoles. Fortunately, the egg stage (typically 1–2 days' duration) is briefer than the tadpole stage (38 days on average in the field: Alford et al. 1995) and, thus, poses a danger for a briefer period. The reluctance of native tadpoles to attack live toad tadpoles (Crossland and Alford 1998; M. Crossland and R. Shine unpublished data) reduces further the vulnerability of frog tadpoles to toad larvae. Not surprisingly, most cases of mortality of frog tadpoles following toad breeding occur immediately postspawning and are due to predation on toad eggs rather than larvae (Crossland et al. 2008). The low toxin levels of small metamorph toads also reduce predator vulnerability; even small native predators that ingest these tiny anurans are at less risk than if they had consumed eggs or larger metamorphs. The defensive toxins reach their minimum around metamorphosis, at the same time as the transition from aquatic to terrestrial life reduces the toad's locomotor ability (Arnold and Wassersug 1978). In combination, metamorphosis is likely to be a critical time in the toad's life history, when both of its usual defenses (fleeing from danger and poisoning predators) are ineffective. The toad's

small body size at metamorphosis and high ratio of surface area to volume add further risk through high rates of desiccation (Cohen and Alford 1993; Child et al. 2008). The end result is that attempts to control populations of this invasive anuran may usefully target the vulnerable and relatively undefended metamorphs, although density-dependent population processes may weaken the effectiveness of this strategy (Cohen and Alford 1993; Alford et al. 1995).

Many methods have been proposed to reduce population densities of cane toads in Australia (Shine et al. 2006). Because these proposals differ in the life history stage targeted for control, the ontogenetic shift in toxicity in toads means that these methods also will differ in their probable effects on native fauna. For example, control of toad populations by introducing sterile males (Mahony and Clulow 2006) would result in eggs remaining in water bodies until they decomposed, prolonging the egg stage and thus increasing the risks of fatal encounter for local native aquatic fauna. In contrast, controlling cane toads by genetic manipulations that kill toad tadpoles prior to metamorphosis (Pallister et al. 2006) would not affect the egg stage but instead would increase the numbers of dead tadpoles. Because frog tadpoles in this system are scavengers rather than predators of live anuran larvae, the dead toad tadpoles would pose a greater toxic risk to native tadpoles than would live toad larvae. However, if mortality is delayed until shortly before metamorphosis, toxin levels (and thus danger to native tadpoles) will be low. Nonetheless, late-stage toad tadpoles are highly toxic to some invertebrate predators (Crossland 1998) and this method of toad control might impose collateral damage on aquatic invertebrates. Reducing toad populations by increasing rates of metamorph mortality (e.g., via parasites—Kelehear 2007) may have less effect on native predators (or at least, vertebrate predators). Understanding ontogenetic shifts in toxin content and hence in predator vulnerability may help in predicting the ecological impacts of alternative approaches to toad control.

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Individual Discrimination by Odors in Sibling Prairie Voles (*Microtus ochrogaster*)

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Abstract The habituation–discrimination paradigm has been used widely to demonstrate that animals can detect individually distinctive odors of unfamiliar conspecifics. By using a modification of the habituation–discrimination technique, Todrank et al. (Anim Behav 55:377–386, 1998) found that golden hamsters discriminate between the individual odors of their own familiar brothers but cannot discriminate between the odors of two siblings that are unrelated and unfamiliar to the subject. This suggested that previous evidence showing that animals could discriminate between the odors of individuals actually may have demonstrated the ability to discriminate between genetically unrelated conspecifics (i.e., members of different families). To test this possibility, we conducted habituation–discrimination experiments with prairie voles, *Microtus ochrogaster*. Voles were tested under three conditions: subject and both targets were unrelated and unfamiliar; subject and both targets were brothers and familiar; subject was unrelated and unfamiliar to targets, but targets were brothers. In all cases, voles discriminated between the two individual odors. Thus, prairie voles can discriminate individual differences between the odors of brothers and they do not have to have previous experience with the conspecifics in order for discrimination to occur. The theoretical importance of these results is discussed.

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Introduction

In many vertebrate species, chemical communication plays a role in social recognition (e.g., Brown 1979; Zenuto et al. 2004; Fernandez-Vargas et al. 2008). Individually distinctive odors particularly have been well studied in rodents and are believed to serve multiple functions that can be critically important to social dynamics (Halpin 1980, 1986).

The chemical composition of chemosensory social signals has been studied in only a few species of rodents. Most signals appear to be composed of a mix of compounds. However, there is sufficient interindividual variation in the relative proportion of the compounds to code for individually distinctive chemosensory cues (Singer et al. 1997; Weidong et al. 1999).

The habituation–discrimination paradigm (Schultze-Westrum 1969; Halpin 1974) has been used widely to investigate the occurrence of individually distinctive odors and the ability of conspecifics to discriminate such individual differences. With this paradigm, it has been demonstrated repeatedly that in rodents and other taxa, subjects reliably distinguish among the odors of conspecifics. A variant technique (often referred to as "habituation–dishabituation") also has been commonly used (Todrank et al. 1998, 1999).

The habituation–discrimination paradigm has two components. During the habituation phase, the subject is presented with the odor of one conspecific (odor A) until the subject habituates to this odor. During the subsequent discrimination phase, the subject is simultaneously presented with odor A and odor from a second conspecific (odor B).

The time spent investigating odors B and A is compared. The assumption is that if the subject cannot distinguish a difference, then it should spend equal time investigating each. If, however, the subject can detect a difference between the odors, then it should spend significantly more time investigating the novel odor B.

Todrank et al. (1998), used habituation–dishabituation to study golden hamsters, *Mesocricetus auratus*. The rationale and mechanisms of this technique are identical to those of habituation–discrimination, but subjects are habituated by a series of brief exposures to odor A and are then presented with odor B. The time spent investigating odor B then is compared to the time spent investigating odor A during the last habituation exposure. Todrank et al. (1998) found that golden hamsters discriminate among the individual odors of their own familiar brothers but cannot discriminate between the odors of two siblings that are unrelated and unfamiliar to the subject. They concluded that the odors of genetically related individuals (i.e., siblings) are so similar that subjects without prior social experience with the odor donors are unable to discriminate between them.

Prior to Todrank et al. (1998), all studies that used these two paradigms had tested odors from conspecifics that were genetically unrelated to one another and both unrelated and unfamiliar to the subject. For example, tests with both of these methodologies demonstrated that golden hamsters discriminate between the odors of unrelated and unfamiliar conspecifics (Johnston et al. 1993; Tang-Martínez et al. 1993).

The results of Todrank et al. (1998) raised an interesting but troubling possibility that in all previous studies that used the two techniques, subjects had been able to distinguish between the odors of two conspecifics only because the two targets were genetically unrelated (i.e., they came from different families). Therefore, one interpretation would be that subjects in previous studies were discriminating between family-related cues rather than between individual ones. If this were the case, siblings might be producing such similar "family odors" that a difference could not be detected by the subject in the absence of previous social familiarity (Todrank et al. 1999).

Such a conclusion opens to question a large body of literature on individually distinctive odors and their putative functions. If animals, in fact, cannot distinguish between the odors of siblings, we need to reevaluate the role of individual odors in social group dynamics and in important evolutionary processes such as kin selection, reciprocal altruism, and mate choice. This possibility makes it imperative that the Todrank et al. (1998) experiments be replicated by using other species and methodologies.

We examined this question by using prairie voles (*Microtus ochrogaster*). Prairie voles are highly social, biparental, monogamous rodents that often live in extended family groups and may remain in contact with siblings even after dispersal (Getz et al. 1993, 1994; Getz and Carter 1996). Newman and Halpin (1988) previously demonstrated that *M. ochrogaster* can discriminate between the odors of two unrelated and unfamiliar conspecifics.

In this study, we hypothesized that prairie voles would be able to distinguish odor differences even between siblings and that the results obtained by Todrank et al. (1998) may not be generalized to all rodents. We now report that male prairie vole can, in fact, discriminate between the odors of (a) their own brothers and (b) two brothers that are unrelated to the subject and with whom he has had no prior experience. This study does not address kin recognition per se or its mechanisms. These questions already have been investigated in prairie voles by using cross-fostering techniques (Gavish et al. 1984; Paz y Miño and Tang-Martínez 1999).

Methods

General Subjects and odor donors came from the vole colony of the University of Missouri-St. Louis. The colony was maintained on a 12:12 h L/D cycle with food (5001 Rodent Diet by Lab Diet) and water provided ad libitum. Voles were weaned at 21 days of age and housed with their siblings in plastic cages (20×23×45 cm) with wood shavings (Aspen Shavings by NEPCO) and paperchip (Cellu-Dri by Shepherd Specialty Papers). All subjects and odor donors were males, at least 40 days old, sexually naïve, and had not been used in other experiments.

Experiments were performed in a room separate from the housing area and conducted during the light phase of the cycle. Each odor donor was single-housed in a plastic cage (15×20×26 cm) with wood shavings for 3 days prior to the trial. Between tests, petri dishes and terraria were washed thoroughly with soap and hot water and rinsed with ethanol. Investigators washed their hands between tests and used latex gloves (American Health Products) to handle Petri dishes and terraria.

We followed the habituation–discrimination method (Halpin 1974; Newman and Halpin 1988). For each trial, the subject was introduced into an empty glass terrarium (30×50×25 cm) and allowed a 5-min acclimation period. During this time, we filled polycarbonate petri dishes (9.5 cm diam) with bedding from cages of the odor donors, taking only dry bedding from throughout the cage. Petri dishes had approximately 60 1-mm holes drilled in both the top and bottom of the dish to allow the subject to smell the contents. Two dishes were filled from the cage of one donor (A) and one dish was filled from the cage of the other donor (B) at the same time.

Following the 5-min acclimation, one Petri dish from donor A was taped to the inside of the terrarium, approximately 5 cm above the floor and in the center of the back wall. For 5 min, we used a stopwatch to record the number of

seconds that the subject spent investigating the dish. Investigation was recorded only when the subject was sniffing within 1 cm of the top surface of the dish. Chewing, climbing, or leaning against the dish were not counted as investigation. After this 5-min period, the subject was left in the terrarium with the same dish of bedding for 50 min; we then recorded investigation for another 5 min. These two 5-min periods, and the 50 min in between, constitute a 1-h habituation period. Habituation was inferred if the subject spent less time investigating the dish during the last 5 min as compared to the first 5 min.

At the end of the 1-h habituation period, the dish used during habituation was removed. Immediately, two new dishes, one containing bedding from odor donor A and the other from odor donor B, were introduced and taped at opposite ends of the terrarium, approximately 5 cm above the floor, with one dish in the center of each side wall. The position (i.e., side of the terrarium) in which dishes containing odors A and B were placed was random across trials. A fresh dish from donor A was used during this phase to control for the possibility that the subject's own odors or markings (e.g., from chewing the dish) would affect the subject's responses. However, since this dish had been prepared at the same time as the original dish from odor donor A and the dish from odor donor B, the two odors used during discrimination were of equal "freshness."

The discrimination phase lasted 10 min, and the time spent investigating each dish was recorded on separate stopwatches. All experiments were performed blind: the dishes used during the discrimination phase were labeled on the bottom prior to the start of the habituation period, the labels were covered, and the dishes shuffled; labels were not rechecked until the end of the discrimination phase. Additionally, in some trials, the position of dishes was assigned by an assistant. Thus, the experimenter was never aware which dish contained odor A or odor B until the trials had been completed and all data had been collected.

The positions of the dishes were changed from the back wall of the terrarium during the habituation phase to the two side walls during the discrimination phase to control for any habituation that might have occurred to the location of the odor source. Thus, during the discrimination phase, both odors were in a novel location, and any differential responses to the two odors by the subject must have been due to perceived differences in the odors rather than to their location.

Experiment 1: (All Unrelated) Our objective was to verify a previous finding (Newman and Halpin 1988) that male voles can discriminate between the odors of two conspecifics. The subject was unrelated to, and inexperienced with, the odor donors, which were also unrelated to one another.

Subjects were 16 males from 16 litters born to 13 breeding pairs (in only two cases did two subjects, used in different tests, come from the same litter). Odor donors were 36 males from 33 litters born to 20 breeding pairs. In three cases, two odor donors came from the same litter but they always were used in different trials; no odor donor was ever paired with the same subject or odor donor in more than one trial. No animal was used as a subject in more than one trial, and no subject was used as an odor donor in other trials.

Experiment 2: (All Related) Here, we tested the ability of the subject to discriminate between the odors of two brothers from his own litter (i.e., subject and odor donors were siblings and socially familiar with one another). Voles were housed together until the odor donors were separated into individual cages 3 days before the trial, as described above.

Subjects were 16 males from 16 litters born to 13 different breeding pairs; odor donors were two brothers of the subject. No animal was used as a subject in more than one trial nor were any subjects used as odor donors in other trials.

Experiment 3: (Odor Donors Related) Odor donors in this experiment were brothers, but they were not related to the subject and he had no prior experience with them. Thus, we tested whether the subject could discriminate between the odors of two animals that were related to each other to the same degree as the odor donors in the second experiment, but who were not related or familiar to the subject.

Subjects were 13 males from 12 litters born to nine different breeding pairs. The odor donors were 13 pairs of brothers from seven breeding pairs. No animal was used as a subject in more than one trial nor were any subjects used as odor donors in other trials.

Statistical Analyses Habituation was determined by comparing time spent investigating odor A during the first and last 5 min of the habituation phase. Since habituation should cause a decrease in investigation over time, these data were analyzed with one-tailed *Wilcoxon signed rank tests* for matched pairs (Siegel 1956).

The results of the discrimination phase were analyzed by comparing time spent investigating the familiar odor (A) and novel odor (B). Since habituation to odor A predicted that more time should be spent investigating odor B (if the two odors are different), one-tailed *Wilcoxon signed rank tests* for matched pairs were used to compare differences. The critical level of significance was set at $P<0.05$.

All aspects of the experimental design were approved by the IACUC of the University of Missouri-St. Louis and conform to USA laws.

Results

In all experiments, the subjects demonstrated habituation to the odor presented during the habituation phase by

decreasing the amount of investigation (given in seconds± S. E.) from the first 5 min to the last 5 min of the habituation period (Experiment 1: N=16, T=3, P<0.005; means=49.7±8.3 vs. 19.4±7.9. Experiment 2: N=16, T=0, P<0.005; means=39.9±6.9 vs. 1.0±0.5. Experiment 3: N=13, T=13, P=0.01; means=42.1±6.0 vs. 21.2±5.1).

In Experiment 1 (All Unrelated), subjects spent more time (given in seconds±S. E.) investigating the novel odor as compared to the odor to which they had habituated (N=16, T=23, P<0.01; mean for A=32.3±4.9, mean for B=50.0± 7.8; 13 of the 16 subjects spent more time investigating the novel odor B). This indicates that the subjects were able to distinguish between the odors of individuals who were unrelated to each other and to the subjects.

In Experiment 2 (All Related), the subjects spent more time investigating the second odor as compared to the odor to which they had become habituated (N=16, T=30, P<0.025; mean for A=27.6±4.9, mean for B=34.8±5.0; despite some overlap of standard error bars, 12 of the 16 subjects spent more time investigating the novel odor B). In other words, subjects were able to distinguish between the odors of their own two familiar brothers, although there was a smaller difference in mean time spent sniffing the two odors as compared to the mean differences in the other two experiments.

In Experiment 3 (Odor Donors Related), the subjects also were able to distinguish between the two odors (N=13, T= 17, P<0.025; mean for A=28.7±5.6, mean for B=44.7±3.5; 10 of 13 subjects spent more time investigating the novel odor B), and more time was spent investigating the novel odor as compared to the habituated odor.

Discussion

We found that subjects discriminated between odor donors that were unrelated to each other and between donors that were familiar brothers of the subject. However, while Todrank et al. (1998) reported that golden hamsters could not distinguish between the odors of brothers that were neither related nor familiar to the subject, we found that prairie voles can make this discrimination. Even if brothers share similar odors because of their genetic relatedness (odor–genes covariance sensu Todrank and Heth 2003), prairie voles discriminate individual differences in these odors. Our findings also confirm that the results of previous studies, on a variety of species (e.g., Halpin 1974, 1980, 1986; Newman and Halpin 1988; Johnston et al. 1993; Tang-Martínez et al. 1993), demonstrated true individual odor discrimination rather than just discrimination of family-related differences.

The methodological differences between the habituation–discrimination protocol used in this experiment and the habituation–dishabituation technique used by Todrank et al. (1998) may be responsible for our different results. Both techniques are used commonly and are considered sufficiently powerful to reveal the existence of individual odors in most situations. However, based on our results, we propose that when the odors of individuals are likely to be extremely similar because of kinship (as in the case of siblings), the habituation–discrimination method may be more sensitive and should be explored before concluding that individually distinctive odors either do not exist or cannot be discriminated by subjects.

This conclusion is buttressed by the results of Jonhston and Peng (2000) on the role of the vomeronasal organ in the ability of golden hamsters to detect individual differences in conspecific odors. After conducting experiments in which the novel odor (B) was presented by itself during the test phase, and others in which odors A and B were presented together, they concluded that simultaneous presentation enhances the subjects' ability to discriminate. Unpublished studies from our laboratory, comparing the two methods, also support this conclusion.

The habituation–discrimination technique is likely to be more sensitive for two reasons. First, the habituation period consists of one long exposure to the odor of the first individual rather than to small numbers of brief repeated exposures. The longer exposure may result in more robust learning and habituation by the subject. Secondly, the simultaneous presentation of the habituated and novel odors allows the subjects to compare directly the two odors rather than having to rely only on the memory of the habituated odor (Jonhston and Peng 2000).

The social ecology of voles and hamsters also may explain the differences between our results and those of Todrank et al. (1998). Specifically, high levels of sociality can be expected to affect the abilities of animals to make fine discriminations among conspecific cues. Golden hamsters are solitary and highly aggressive (Lawlor 1963; Payne and Swanson 1970). In contrast, prairie voles are highly social and often live in extended family groups (Getz et al. 1993, 1994; Getz and Carter 1996). Accordingly, selection likely favored prairie voles that were able to make fine odor discriminations among relatives, an ability that may allow them also to perceive subtle individual differences in the odor cues of other conspecifics. For solitary golden hamsters, it is unlikely that there was strong selection for the ability to discriminate such small differences in conspecific cues. We suggest that the results obtained with golden hamsters (Todrank et al. 1998) cannot be generalized to social species that have complex social organizations.

Differences in the genetic history of the two test populations undoubtedly are also important. All golden hamsters used in laboratories in the USA are descended from one male and two females collected in the field in

1930 (Adler 1948; Bunnel et al. 1977). Thus, laboratory hamsters are highly inbred and genetically similar to one another. By contrast, the vole colony used in our experiments was created from more than 20 voles trapped in the field. To avoid excessive inbreeding, new animals (also field-trapped) were added to the lab population approximately every 2 years. Given that odor differences are correlated with genetic differences (i.e., "odor-genes covariance": Todrank and Heth 2003), the odors of sibling voles certainly are more individually distinctive than the odors of sibling golden hamsters from laboratory colonies. As a result, voles apparently do not need to have prior experience with the distinctive odors of closely related conspecifics before they can distinguish them.

The results of Todrank et al. (1998) are particularly important because they demonstrate that extremely close genetic relatedness may impact the ability of animals to distinguish individual odors of conspecifics. Our data indicate that species or populations with different social, ecological, and/or genetic characteristics also may differ in their ability to perceive subtle differences in odors. Moreover, when differences in individual odors become more tenuous (e.g., when two individuals are closely related genetically), animals should have more difficulty discriminating between these odors. By modifying experimental paradigms to facilitate learning or comparison of conspecific odors (e.g., as in habituation–discrimination), we increase the probability that subjects will be able to distinguish even between the odors of close genetic relatives.

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Altered Olfactory Receptor Neuron Responsiveness Is Correlated with a Shift in Behavioral Response in an Evolved Colony of the Cabbage Looper Moth, *Trichoplusia ni*

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Abstract There is little understanding of how sex pheromone blends might change during speciation events. For the cabbage looper, *Trichoplusia ni*, there is a mutant laboratory strain that has exhibited characteristics of a shift to a new pheromone blend. Mutant females produce a blend that is significantly different from wild-type females in having a much higher proportion of a minor pheromone component and lower quantity of the major component. Males in this colony have changed over the years to become more broadly tuned and fly upwind equally well to both the wild-type and mutant female pheromone blends. They also exhibit reduced overall sensitivity to pheromone, flying upwind to either blend at a lower success rate than is typical when wild-type males respond to the wild-type blend. Using single-cell recordings, we examined the olfactory receptor neurons (ORNs) of males from evolved and wild-type colonies for evidence of changes in response characteristics that might explain the above-described behavioral evolution. We found that in evolved-colony males the ORNs tuned to the major sex pheromone component exhibited a somewhat lower responsiveness to that compound than the ORNs of wild-type males. In addition, the minor pheromone component, emitted at excessively high rates by mutant females, elicited a drastically reduced ORN responsiveness in evolved-colony males compared to wild-type males. This alteration in ORN responsiveness may be responsible for allowing evolved males to tolerate the excessive amounts of the minor pheromone component in the mutant female blend, which would normally antagonize the upwind flight of unevolved males. Thus, peripheral olfactory alterations have occurred in *T. ni* males that are correlated with the evolution of the more broadly tuned, but less sensitive, behavioral response profile.

Keywords Lepidoptera · Electrophysiology · Pheromone · Evolution · Behavior · Olfaction · Antenna

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Introduction

The nature of pheromonal changes related to speciation has been speculated upon frequently (Phelan 1992; Baker 2002, 2008; Symonds and Elgar 2004; Symonds and Wertheim 2005). It has been particularly difficult to pinpoint what initial mutations to olfactory systems might occur that provide the raw material upon which selection could operate. According to the "asymmetric tracking" hypothesis (Phelan 1992), the nonlimiting sex (usually males) that experiences stronger selection will more strongly track changes that occur in the limiting sex (usually females). For systems in which females emit sex pheromones, this asymmetry manifests itself in high between-individual variation in emitted pheromone blend composition, while

males have high within-individual variation in behavioral response profiles to these blends that bracket the pheromone composition of most females in the population (Löfstedt 1990). Therefore, shifts in female pheromone blends might initially require that males in the population broaden their response profiles to include mutant female pheromone blends as well as the wild-type female blends.

Pheromone olfactory pathways logically can be inferred as playing a key role in allowing broad pheromone blend responsiveness of males in this first stage of the asymmetric tracking process. Indeed, olfactory receptor neuron (ORN) tuning profiles have been implicated in the broader responsiveness of "rare" males in *Ostrinia nubilalis* and *Ostrinia furnacalis* that fly upwind equally well to the quite different pheromone blends emitted by females of these two species (Roelofs et al. 2002; Linn et al. 2003, 2007; Domingue et al. 2007a, b).

A system that is particularly well suited for exploring the olfactory changes possibly involved in broader behavioral response related to speciation is the sex pheromone behavior of the cabbage looper moth, *Trichoplusia ni*. This species uses (*Z*)-7-dodecenyl acetate (Z7-12:OAc) as its major pheromone component along with five minor pheromone components (Bjostad et al. 1984; Linn et al. 1984). In 1990, a mutant female phenotype, caused by a single gene mutation, was reported to produce a 1:2 ratio of the minor pheromone component (*Z*)-9-tetradecenyl acetate (Z9-14:OAc) to Z7-12:OAc compared to the normal wild-type proportion of 1:100 (Haynes and Hunt 1990). An "evolved" colony then was developed in which all females displayed this inherited mutant trait, which is controlled by a major autosomal gene (Haynes and Hunt 1990).

Initially, males from this mutant female colony were like wild-type males, in that they were more likely to fly upwind to and reach the source of the wild-type female pheromone blend than they were to the mutant female blend. Then, between generations 10 and 25, males from the evolved colony began to fly to the mutant female blend more frequently while retaining the same upwind flight success rate in response to the wild-type female blend. The success rate of upwind flight and source contact by males from the evolved colony in response to either the mutant or the wild-type blend became equal at generation 37, e.g., after approximately 3 years (Liu and Haynes 1994; Haynes 1997).

In an early attempt to see whether the ORNs of evolved-colony and wild-type males differed in their abundance or response profiles, recordings were performed on male sensilla from generations 14–17 (Todd et al. 1992). Most sensilla (type I) were found to house a larger-spike-size ORN responding primarily to Z7-12:OAc, with sometimes a lower-frequency response to (*Z*)-7-tetradecenyl acetate (Z7-14:OAc) and a smaller-spike-size ORN responding to (*Z*)-7-dodecen-1-ol (Z7-12:OH). There were also sensilla (type II) in which one ORN responded strongly to Z9-14:OAc. There was a rare sensillum type (type III) housing one ORN primarily tuned to another minor pheromone component, Z7-14:OAc. The degree to which behavioral evolution of the males had begun at this point is not clear, and physiologically tested males also were not behaviorally characterized. There were no obvious differences in the relative proportions of sensillum types in the two colonies. However, the firing frequencies on ORNs responsive to Z9-14:OAc appeared to be lower in evolved-colony vs. wild-type males. If the tendency to respond behaviorally to pheromone blends with greater proportions of Z9-14:OAc had begun at this point, a lower responsiveness to Z9-14:OAc could have facilitated such a response, assuming that the relative firing ratios of type I and type II ORNs influence behavior. In the ensuing 2 years, the evolved-colony males clearly began to exhibit the broad behavioral tuning consistent with the asymmetric tracking model (Phelan 1992). A behavioral tendency to fly successfully upwind to mutant- and wild-type female pheromone blends had become fixed in the population (Liu and Haynes 1994; Haynes 1997).

We checked the ORNs of these evolved-colony males vs. wild-type males in 1996 (Todd et al., previously unpublished) and again more thoroughly in 2007 in order to refine our knowledge about what, if any, changes in the peripheral receptor tuning profiles may have occurred that could explain the behavioral evolution. Recently, it was reported (Hemmann et al. 2008) that the more broadly behaviorally receptive males from the evolved colony also are less sensitive in response to either pheromone blend than wild-type males are to the wild-type blend. Thus, higher doses of either blend are required before evolved-colony males fly upwind. Here, we also examined whether changes in the responsiveness of the ORNs might have occurred that are consistent with this reduced behavioral sensitivity.

Methods and Materials

Electrophysiological sampling of wild-type and evolved-colony males has occurred on separate occasions at three historical points, in generations 14–17 (1990, see Todd et al. 1992), in generations 86–90 (1996), and finally in about the 220th generation (2007). At the different time points, experiments were performed by different analysts by using slightly different methodologies. The methodology used in 1990 has been described previously (Todd et al. 1992). It was not until the recent 2007 experiments that we developed and performed a highly replicated and well-controlled experimental protocol to compare the relevant characteristics of the ORNs responsive to Z9-14:OAc and

Z7-12:OAc in wild-type and evolved-colony males. Thus, we primarily describe the methodology for this most recent experiment, noting how the previous experiments differed.

Insects The wild-type and evolved colonies were originally derived from a colony collected in Riverside, CA, USA (Haynes and Hunt 1990). Both colonies were used in the 1990 physiology experiments. In 1996 and 2007, the same evolved colony was used, but the wild-type colony of *T. ni* derived from a colony maintained at the US Department of Agriculture-Agricultural Research Service Insect Attractants, Behavior, and Basic Biology Research Laboratory in Gainesville, FL, USA (Hemmann et al. 2008). Colonies were reared on a pinto-bean-based diet (Shorey and Hale 1965), at ambient room temperature, humidity, and light conditions. Male pupae of each colony were shipped from Kentucky to California (1990), Iowa (1996), or Pennsylvania (2007) for physiological analysis.

For the 2007 experiment, the source of the evolved-colony and wild-type males was withheld from the physiological analyst until the experiment was completed. Pupae and emerging adults were incubated at 25°C, in plastic containers with a Petri dish full of cotton saturated with sugar water at a 16:8 h L:D photoperiod. Different containers were used to separate males emerging within each day, such that we could ensure that all males sampled were between 2 and 7 days old when analyzed.

Single-Cell Electrophysiology ORN responses were recorded from trichoid sensilla by using the cut sensillum technique (Kaissling 1974; van der Pers and den Otter 1978). Individual antennae were excised from the head and placed in a saline-filled recording electrode including a silver wire. When possible, recordings were made from both antennae of an insect. Each antenna was positioned with a micromanipulator such that a single trichoid sensillum rested on the tip of a vertically positioned tungsten knife. A second horizontally oriented glass knife, controlled with another micromanipulator, was used to cut the sensillum tip. The cut sensillum then was contacted and surrounded by a saline-filled glass micropipette containing a silver recording electrode.

A stream of purified humidified air blew continuously over the antenna (10 ml/s) through a 25-cm-long glass tube (8-mm inner diameter (ID)), the end of which was placed 2 cm from the antenna. A 50-ms air pulse at a 40-ml/s flow rate was injected through the odor cartridge and into the airstream by using a stimulus flow-controller device (SFC-2, Syntech). Linear flow through the air-stream was ~0.3 m/s. The AC signal from the recording electrode passed through a built-in amplifier (DAM50, World Precision Instruments, Sarasota, FL, USA) and recorded with a computer. An external loudspeaker coupled with computer software (Syntech Autospike v.32; Syntech, Hilversum, The Netherlands) allowed visual and auditory monitoring of neural activity as it was recorded. In the 1996 and 2007 experiments, we observed little spontaneous background activity, typically less than one spike per second (Fig. 1). To measure spike frequency, Syntech software was used to count the number of spikes occurring within 300 ms of the first appearance of a spike. If there were less than two spikes within a second of a puff, the response was considered negative.

ORN Classes in 1990 and 1996 A random search protocol for sampling the sensilla was performed at the first two time points in 1990 and 1996. In 2007, a different analyst knew more about the spatial distribution of sensillum types, which

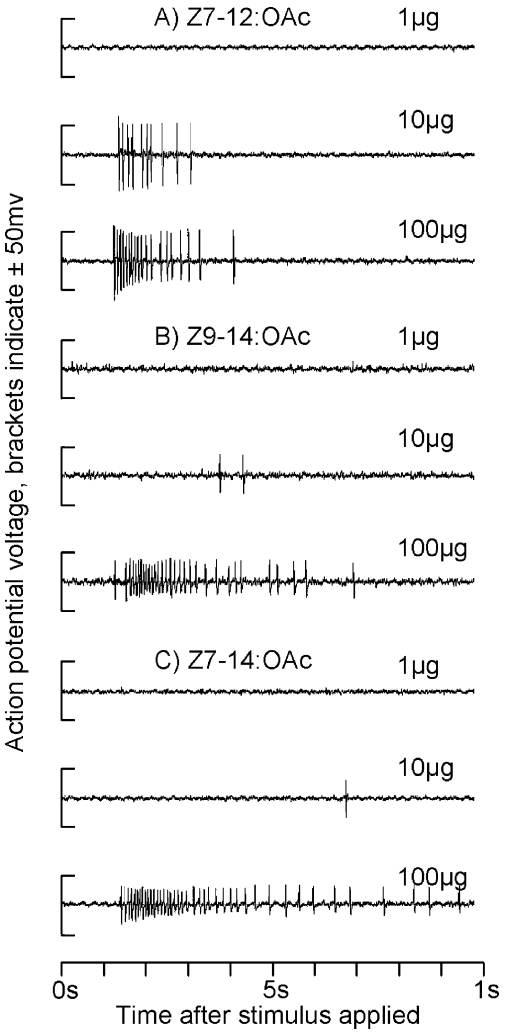

Fig. 1 Dose–response series (0.1-µg dose excluded) from ORNs housed within the three sensillum types commonly observed in *Trichoplusia ni*. The compounds inducing responses in these ORNs include Z7-12:OAc (*A*), Z9-14:OAc (*B*), and Z7-14:OAc (*C*), respectively. This series were obtained from different sensilla of the same male

precluded the same random sampling scheme (see Grant et al. 1998). The 1996 experiment followed the previously described protocol for the 1990 study (Todd et al. 1992). Compounds screened included Z7-12:OAc, Z7-12:OH, Z7-14:OAc, Z9-14:OAc, 12:OAc, 11–12:OAc, and Z5-12:OAc. Sensillum types were assigned based on responses to Z7-12:OAc and Z7-12:OH (type I), Z9-14:OAc (type II), and Z7-14:OAc (type III). The other minor pheromone components usually only weakly stimulate ORNs sensitive to these other compounds. In all colonies, there also have been <5% sensilla with ORNs broadly tuned to many compounds, which are not considered in our analyses. Thus, our current approach reduces the potential number of sensillum types compared to what was delineated in the 1990 study (Todd et al. 1992).

Comparisons of the relative proportion of sensilla belonging to each of the three sensillum types in the evolved and wild-type colonies in 1990 and 1996 were made by using logistic regression including *colony* (evolved vs. wild-type) and *year* (1990 vs. 1996) as factors that might explain the frequency of sensillum types observed. PROC LOGISTIC in SAS (version 9.3) was use to perform this analysis.

Dose–Response Relationships Dose–response relationships were investigated in 1996 and more thoroughly in 2007. In both cases, we made pheromone cartridges from dilutions of Z7-12:OAc, Z9-14:OAc, and Z7-14:OAc in high-performance liquid chromatography (HPLC)-grade hexane. For each concentration (0.01, 0.1, 1, or 10 μg/μl), 10 μl was pipetted onto a 0.5 × 2.0-cm^2 filter paper strip held in a 15-cm-long Pasteur pipette odor cartridge. The filter paper loadings thus were 0.1, 1, 10, or 100 μg, a range that includes the 10-μg dose used successfully previously with the cut-tip method (Todd et al. 1992). The same cartridges were used for all recordings over an approximately 1-month period within a given year.

After a single stimulation with one such cartridge, there was at least a 30-s delay before further testing. For a typical sensillum, we began by administering the three compounds at the lowest concentration in random order. We then continued at each successively higher concentration by using the same order of compounds. When it became clear which sensillum type we had encountered, we usually completed the dose–response curve for the relevant compound only. For verification of the sensillum type, the other two compounds were puffed at the highest concentration at the end of the experiment. This protocol was also important to prevent biases in the pretest exposure of these compounds to other sensilla because we usually sampled multiple sensilla per antenna.

A total of 25 type I sensilla and 12 type II sensilla were sampled from wild-type males in 1996. During this same period, 12 type I sensilla and four type II sensilla were sampled from evolved-colony males. In the 2007 experiment, sensilla sampled included 149 type I, 36 type II, and 62 type III from wild-type males. We also sampled 88 type I, 33 type II, and 11 type III sensilla from evolved-colony males in 2007.

Additional precautions were employed for the 2007 experiment. The larger sample in 2007 allowed a more thorough exploration of statistical differences between the ORN characteristics of ORNs tuned to Z7-12:OAc vs. Z9-14:OAc in the populations, which might be relevant to their behavioral differences. By using the pattern of the distribution of sensillum types described by Grant et al. (1998), sampling was focused on the medial portion of the antenna where sensilla housing ORNs responsive to Z7-12:OAc and Z9-14:OAc should be found with high frequency. At the beginning of the experiment, sensilla were sampled randomly from this region. Sensilla were encountered with ORNs responsive to Z9-14:OAc much less frequently than those for Z7-12:OAc, and, thus, antennae were sampled as many times as possible in order to ensure that Z9-14:OAc-responsive ORNs were encountered at least once per moth. As the experiment proceeded, the analyst developed the ability to visually distinguish the sensillum types. To maintain an unbiased approach, pairs of ORNs responsive to Z7-12:OAc or Z9-14:OAc were sampled per antennae in random order, before sampling sensilla likely to have ORNs responsive to Z7-14:OAc. Thus, throughout the experiment, we ensured that the order of sampling of sensilla with ORNs responsive to Z7-12:OAc vs. Z9-14:OAc remained random. Also, sampling from the two colonies, coded only by number, was regularly alternated as much as possible, with approximately equal proportions of the moths sampled from the two colonies over each of the 3 weeks of the experiment.

To analyze the data, spike frequency dose–response curves were calculated for mutant vs. wild-type males in the 1996 and 2007 populations. Further hypotheses were tested concerning the response characteristics of Z7-12:OAc and Z9-14:OAc in the 2007 experiment. First, the maximum spike frequencies obtained within each dose series were compared for the ORNs responsive to Z7-12:OAc and Z9-14:OAc in wild-type vs. evolved-colony males. Usually, the greatest response was elicited at 100 μg, but sometimes there was a slight decline at 100 μg after a stronger response at 10 μg. Data were log-transformed to ensure that the distributions were normal and of homogeneous variance. Analysis of variance (ANOVA) was performed, using *ORN type*, *colony*, and *ORN type*×*colony* as factors. The analysis was performed such that per individual rather than per sensillum differences were considered. Thus, the latter two factors were nested within the individual sampled from (*Moth*).

The other hypothesis of interest was whether there were any differences between the colonies with respect to the threshold for responses to Z9-14:OAc and Z7-12:OAc. For this purpose, the minimum concentration eliciting responses to Z9-14:OAc or Z7-12:OAc was noted for all the relevant ORNs. Possible shifts in the threshold response doses were assessed by logistic regression, assuming a cumulative logit model, using the SAS procedure GENMOD. Contrasts were evaluated by comparing the distributions of thresholds of wild-type to evolved-colony males for ORNs in type I or type II sensilla. The interaction effect between *colony* and *ORN type* also was evaluated. It also must be considered that the unbalanced sampling of varying numbers of sensilla across males might bias the results. Thus, rather than using all sensilla without considering such subsampling, we weighed each observation proportionally to the number of sensilla sampled for that moth.

Cartridge Emissions and ORN Spike Frequencies in Type I and Type II Sensilla Compounds emitted from odor cartridges were collected as they were issued from the pipette tip in 25-cm-long glass collection tubes (3-mm ID). The tip of an odor cartridge containing the filter paper strip dosed with one of the pheromone components was inserted into a collection tube, and the connection was sealed with Teflon tape. The collection tube was placed in a container (20 cm long × 3-cm ID) filled with dry ice. The odor cartridge was connected to the flow controller that generated 20-ms puffs of air with the flow set at 15 ml/s through the cartridge. Collection tubes were washed with 30 µl of HPLC-grade hexane containing (*Z*)-10-pentadecenyl acetate (50 pg/µl) as an internal standard. Collected amounts were analyzed by using gas chromatography–mass spectrometry (GC–MS) in selective ion mode. Collected amounts were calculated as mean picogram per puff and corrected for differences in relative abundance of the selected ions relative to the internal standard. Trap breakthrough was checked and confirmed negative for all odor cartridges at the highest dose level by analyzing collected material in a second, in-series-connected, glass collection tube.

Collection protocol was as follows: Z7-12:OAc was collected by using 512 pulses for each dose. Collection of Z9-14:OAc was performed with 4,096 pulses of each dose. Collections were analyzed by using a 30-m DB-1 capillary column. All collection tubes were stored at −20°C prior to analyses by GC–MS.

For the 1996 and 2007 ORN data, ORN firing frequencies were plotted in response to emitted amounts of Z7-12:OAc and Z9-14:OAc from the cartridges. We interpolated the Z7-12:OAc to Z9-14:OAc ORN relative firing ratios in response to emitted amounts of Z7-12:OAc and Z9-14:OAc that had been measured from mutant and wild-type females (Haynes and Hunt 1990) in order to relate the peripheral olfactory input experienced by evolved-colony and wild-type males to their upwind flight behavior when exposed to the evolved-colony and wild-type pheromone blends (Liu and Haynes 1994; Hemmann et al. 2008).

Results

Compared to males in the wild-type colony, the ORNs of evolved-colony males exhibited lower mean spike frequencies in response to Z9-14:OAc and Z7-12:OAc at all concentrations in both 1996 and 2007 (Figs. 2 (A, B) and 3 (A, B)). However, the reduction in spike frequency was much greater for the evolved-colony male ORN response to Z9-14:OAc vs. that to Z7-12:OAc. Lower responsiveness of the Z9-14:OAc ORN in evolved-colony vs. wild-type males had been observed in the 1990 populations, although dose–response curves were not collected and sample sizes were smaller than in 2007 (Todd et al. 1992). In contrast, the 2007 spike frequencies in wild-type vs. evolved-colony males responsive to Z7-14:OAc appeared similar at all odorant doses (Fig. 3 (C)).

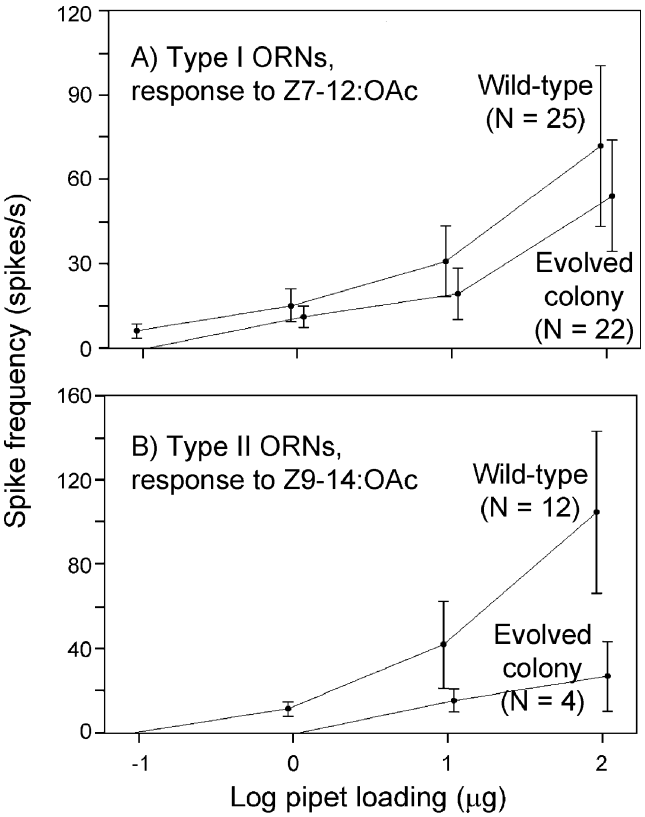

Fig. 2 Dose–response relationships for ORNs of type I (*A*) and type II (*B*) sensilla to their respective ligands Z7-12:OAc and Z9-14:OAc (mean spike frequency+SE). Means reflect pooled data from all individuals in which ORNs were sampled in 1996, between generations 86 and 90

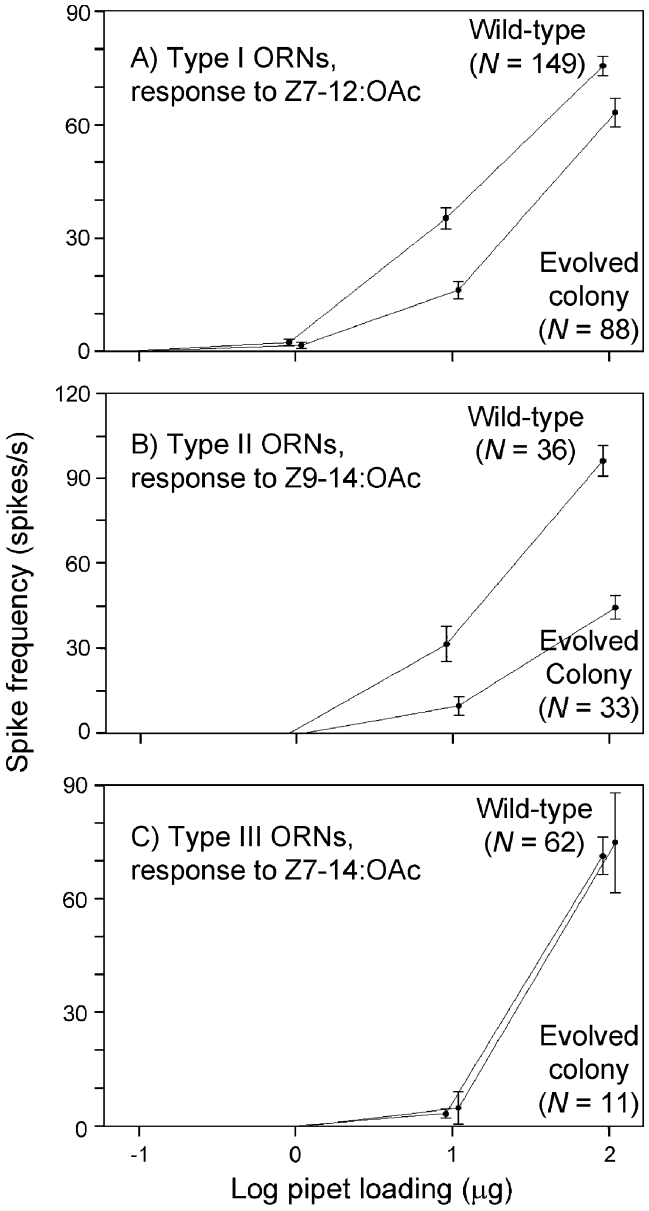

Fig. 3 Dose–response relationships for ORNs of type I (*A*), type II (*B*), and type III (*C*) sensilla to their respective ligands Z7-12:OAc, Z9-14:OAc, and Z7-14:OAc (mean spike frequency+SE). Means reflect pooled data from all individuals in which ORNs were sampled in 2007 (about 220 generations after the colony was initiated)

There were relatively fewer type II vs. type I sensilla located in the random search for evolved-colony vs. wild-type males as indicated by the corresponding significant regression coefficient (Tables 1 and 2). There was no similarly statistically significant relationship between the frequencies of type III vs. type I sensilla with respect to the *colony* effect (Table 2). However, it is also relevant to note that in 2007 we became able to locate any of these three sensillum types based on their consistent location and morphology. After achieving this capability, we could not detect any differences in the frequencies of these sensillum types. In the regression model presented in Table 2, only the *colony* effect is shown. Models also were considered where *year* and *colony*×*year* interactions were considered. In a full model that used all three factors, the model did not converge when the iterative method for obtaining maximum likelihood estimates of the parameters was performed. Models were also considered where either *year* or *colony-by-year* interactions were removed, which in both cases did not exhibit such a lack of convergence. Regardless of the model selected, *colony* was the only effect that was ever significant. Thus, we present the simplest model containing only this effect.

The pattern observed for differences in ORN response characteristics between wild-type and evolved-colony males in the dose–response curves is further reinforced by statistical analysis of the maximum spike frequencies for the Z9-14:OAc- and Z7-12:OAc-responsive neurons in wild-type and evolved-colony males from 2007. The average maximum spike frequency in response to Z9-14:OAc was greater than that for Z7-12:OAc in wild-type males (Fig. 4). This measurement was lower for both compounds in evolved-colony males. However, the reduced maximum spike frequency in evolved-colony males was much greater for responses to Z9-14:OAc than for those to Z7-12:OAc, thus resulting in lower response levels to Z9-14:OAc. The interaction effect indicative of this observation is statistically significant (Table 3).

There were significant differences in the distribution of observed threshold of responses for the evolved-colony and wild-type populations of ORNs for both type I and type II sensilla (Table 4). In both cases, more ORNs have a higher threshold of response to Z7-12:OAc or Z9-14:OAc in evolved-colony male vs. wild-type male sensilla. There was

Table 1 Proportion of sensilla of each ORN type

Sensillum type	Wild-type colony		Evolved colony	
	1990	1996	1990	1996
I (Z7-12:OAc)	77/112 (69%)	58/80 (72%)	83/96 (87%)	51/55 (95%)
II (Z9-14:OAc)	26/112 (23%)	12/80 (15%)	10/96 (10%)	4/55 (7%)
III (Z7-14:OAc)	3/112 (3%)	6/80 (7%)	3/96 (3%)	0/55 (0%)

The colonies were sampled in 1990 (gens. 14–17) AND 1996 (gens. 86–90)

Table 2 Results of logistic regression involving the proportion of sensilla of each ORN type, as described in Table 1

Test[a]	*Wald* X^2	*df*	*P*
Colony	10.7	2	0.005
Colony effect on type I vs. type II frequency	8.72	1	0.003
Colony effect on type I vs. type III frequency	2.59	1	0.107

[a] Test statistics and hypothesis tests are for maximum-likelihood-derived estimates of regression coefficients

no significant interaction effect between population and sensillum type in this analysis.

The amounts emitted per puff from the odor cartridges for a given filter paper loading conformed to previous measurements involving pheromone components that differed in chain length by two carbons (Cossé et al. 1998). The amount per puff of Z9-14:OAc emitted was approximately ten times lower than that of Z7-12:OAc at 1-, 10-, and 100-µg loading, respectively (Fig. 5).

Emitted amounts from cartridges were used not only for calculating ORN response thresholds (Table 4) but also for comparing the Z7-12:OAc to Z9-14:OAc ORN spike frequency ratios of evolved-colony compared to wild-type males in response to the mutant- and wild-type-female-emitted blends (Fig. 6). Amounts of Z7-12:OAc and Z9-14:OAc emitted by wild-type and mutant females (Hunt and Haynes 1990) fell between the emitted cartridge amounts for the 1996 and 2007 ORN data, as shown in Fig. 6 for our 2007 recordings only.

In both 1996 and 2007, wild-type males that, as a group, exhibited good upwind flight in response to their wild-type female pheromone blend (Liu and Haynes 1994; Hemmann et al. 2008) had a slightly greater than 1:1 Z7-12:OAc to Z9-14:OAc ORN firing ratio in response to this blend (Table 5). Similarly, evolved-colony males that had evolved the ability to fly upwind successfully in response to either the mutant or wild-type female blends exhibited ratios of firing of Z7-12:OAc to Z9-14:OAc ORNs that were approximately 1:1 or 3:1, respectively (Table 5). In contrast, in both years, wild-type males challenged to fly upwind in response to the mutant female blend exhibited poor upwind flight (Liu and Haynes 1994; Hemmann et al. 2008). It is of interest that the ORN firing ratio of this group of males in response to the mutant blend was the only one in which the Z7-12:OAc-tuned ORN's contribution to the ratio was only one half that of the Z9-14:OAc-tuned ORN (a 1:2 firing ratio; Table 5).

Discussion

Three aspects of ORNs responsive to Z9-14:OAc and Z7-12:OAc on male *T. ni* antennae were examined that could be relevant to the historical broadening of the behavioral response of evolved-colony males. This response now includes upwind flight to both the unusual pheromone blend produced by mutant females as well as the blend emitted by wild-type females (Haynes and Hunt 1990; Liu and Haynes 1994). These aspects include: (1) the relative abundance of each sensillum type; (2) the response threshold of each ORN within each type of sensillum; and (3) the spike frequencies exhibited by these ORNs in response to a given stimulus. Of these factors, all showed some statistically significant differences between evolved-colony and wild-type populations.

The most obvious difference we observed was the severe reduction in firing frequency of evolved-colony male exhibited by ORNs tuned to Z9-14:OAc (Figs. 2, 3, and 4, Table 3). Evolved-colony males also exhibited significantly reduced sensitivity and firing frequency of the ORN tuned to Z7-12:OAc compared to this type of ORN in wild-type males. However, there was a much greater reduction in responsiveness of the Z9-14:OAc-tuned ORN, underscored by the statistically significant interaction comparing alterations to ORN responsiveness in type I (Z7-12:OAc) vs. type II (Z9-14:OAc) sensilla in evolved-colony vs. wild-type males in 2007 (Fig. 4, Table 3). While the studies of evolved-colony and wild-type males in 1990 (Todd et al. 1992) and 1996 were not replicated and controlled well enough to demonstrate a similar statistically significant

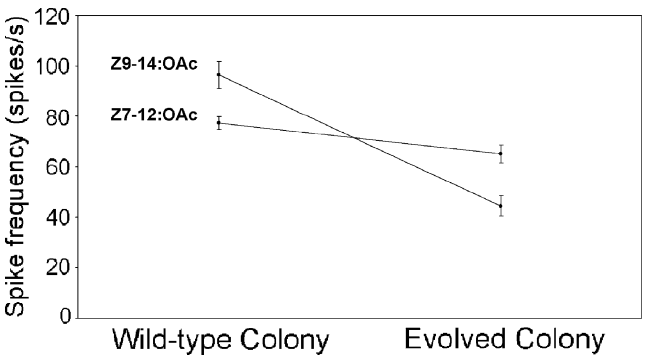

Fig. 4 Average of the maximum ORN response within each dosage series (mean spike frequency+SE) to Z7-12:OAc and Z9-14:OAc in the wild-type and mutant colonies. Means are weighted using per individual variation

Table 3 Analysis of variance testing the effect of the ORN sampled from and colony source as factors in the maximum spike amplitude within each dose–response series

Source	*df*	Sum of squares	Mean square	*F*	*P*
ORN type[a]	1	21.65	21.65	0.29	0.589
Error	248	18,360	74.05		
Colony[b]	1	4,608	4,608	32.51	<0.001
ORN type×colony[b]	1	904.6	904.6	6.38	0.014
Moth (colony)	53	7,511	141.7		

[a] The per sensillum error is used for hypothesis testing

[b] The latter two factors were nested within the individual sampled from (*Moth*), the error which is used for hypothesis testing for these two factors

effect, the trend also can be observed in the 1996 data (Fig. 2) and in 1990 (Todd et al. 1992). In the 1990 study, the ORN spike frequency in type I sensilla screened for response to Z7-12:OAc was lower in evolved-colony than in wild-type males by about 25% (134.6/s vs. 98.8/s). At the same time, a screening of type II ORN response to Z9-14:OAc showed a spike frequency in mutant male ORNs that was about 50% lower in evolved-colony than in wild-type males (142.3/s vs. 72.7/s).

It appears that there has been the evolution of a highly significant dampening of ORN responsiveness to Z9-14:OAc in males from the mutant colony. Thus, a behavioral response to a greater proportion of Z9-14:OAc in the female blend of mutant females is correlated with a diminished physiological response to that same component by the males. This alteration seems to have helped place the ratio of Z7-12:OAc to Z9-14:OAc peripheral inputs within a range acceptable to integrative centers in the brain. Therefore, a simple selective attenuation of a peripheral response to one component may explain the behavioral response.

However, a reduction in sensitivity of the Z9-14:OAc ORN pathway cannot also explain how evolved-colony males have simultaneously retained their ability to fly successfully upwind in response to the wild-type pheromone blend. The Z7-12:OAc to Z9-14:OAc spike frequency ratios in wild-type ORNs exposed to the wild-type female blend range from 1.13:1 to 1.41:1, whereas the ratios of evolved-colony male ORNs flying upwind to wild-type or mutant blends were somewhat lower or higher than this range, respectively. In evolved-colony males exposed to the wild-type blend, the ratio of spiking activity would be more heavily skewed (3:1) toward the Z7-12:OAc ORN than in wild-type males exposed to this same blend. Thus, ORN spike frequency ratios, which are somewhat higher (3:1 for evolved-colony male ORNs to the wild-type blend) or lower (0.83:1 or 1.05:1 for evolved-colony male ORNs to the mutant blend) than the ratio range shown above for the wild-type male ORNs to the wild-type blend, are at least adequate for upwind flight. The poorest upwind flight and source contact was exhibited by wild-type males in response to the mutant blend, and this ratio was low (1:2) and dominated by the activity of the Z9-14:OAc ORN, apparently making it out of the normally acceptable range to promote flight.

It is difficult to reconstruct precisely the sequence of olfactory changes that occurred at the inception of the evolved colony along with the evolution of behavioral responsiveness to both the mutant and wild-type pheromone blends. Many critical tests that would have been

Table 4 Threshold of responses in type I and type II sensilla from 2007

	Weighted threshold frequencies[a]					
	Z7-12:OAc (type I)			Z9-14:OAc (type II)		
Pipette load (μg)	Emission (ng)	Wild	Evolved	Emission (ng)	Wild	Evolved
1	0.0039	3.37	1.39	0.0003	0	0
10	0.54	11.4	17.9	0.016	11.2	7.50
100	8.4	8.20	27.7	0.86	5.83	15.5
Within sensillum[b]		$X^2 = 4.64$	$P < 0.031$		$X^2 = 4.39$	$P = 0.036$
Sensillum type by colony interaction		$X^2 = 0.10$	$P = 0.753$			

[a] Frequencies are weighted proportionally to the number of sensilla sampled per moth

[b] Results from logistic regression involving type I and type II sensillum are included (see text for details)

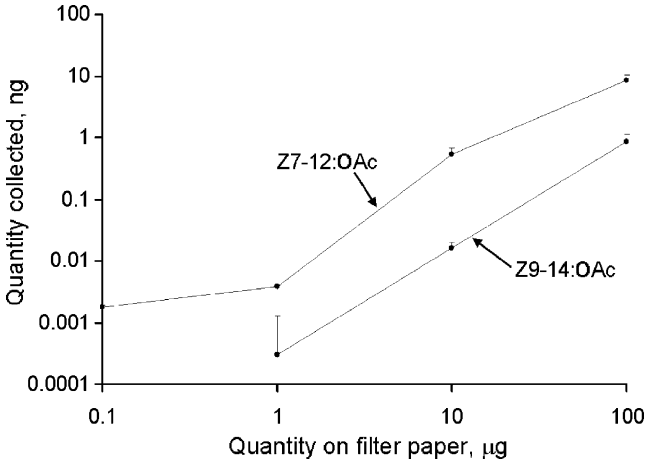

Fig. 5 Relationship between the dosage applied to filter papers in the odor cartridges and the amounts emitted from the cartridges per single puff (mean+SE). Sample sizes vary for each concentration ($N = 2$ for Z7-12:OAc at 1 µg, $N = 3$ for Z7-12:OAc at 0.1 µg, and Z9-14:OAc at 1 µg and 10 µg, $N = 4$ for Z7-12:OAc at 10 µg and 100 µg, and Z9-14:OAc at 100 µg)

useful in the earliest generations did not become apparent until later on. Despite such inherent difficulties, a reasonable historical scenario is beginning to emerge.

At the initiation of the evolved colony line, male ORNs from the first few generations were not sampled to provide a baseline reading of ORN firing frequency ratios. However, because males were randomly selected from the wild-type population to mate with mutant females to initiate the mutant colony, it is reasonable to assume that the ORN firing ratios in response to different pheromone blends did not differ initially between the two colonies, and this is consistent with behavioral observation at the time. A new pattern of behavioral responsiveness to the mutant female blend by males began to evolve between 10 and 25 generations after the colony was founded (Liu and Haynes 1994). Males from this colony have exhibited a shift from an approximately 1:2 Z7-12:OAc to Z9-14:OAc ORN firing ratio in response to the mutant pheromone blend to a 1:1 ratio in response to this mutant blend. Concomitantly, a ratio of nearly 3:1 of Z7-12:OAc to Z9-14:OAc ORN firing in response to the wild-type blend was established as a result of this shift in ORN responsiveness. This ratio likewise is coincident with the retention of adequate levels of upwind flight by evolved-colony males to the wild-type female blend. Although we can provide only strong statistically confirmation of this physiological alteration from more recently employed experiments, it is likely to have occurred by 1990 (generations 14–17), when limited sampling pointed to this trend (Todd et al. 1992).

We caution that it is not clear if the physiological alteration is a direct cause of the behavioral evolution of the mutant colony population or is one in addition to other potentially important factors. These physiological assays of earlier generations had sample-size limitations and were not experimentally linked to behavioral observations, while neither trait was likely to be fixed in the population. Further experimental inquiries into the degree of genetic linkage between the mutant behavior, the ORN firing ratio alteration observed here, and perhaps yet unknown factors are still possible and could contribute to understanding how this novel behavior evolved. It also would be interesting to investigate the potential linkage of the female pheromone and male ORN and behavioral response traits. With these caveats, the current focus on peripheral ORN response shifts do not preclude the role of olfactory alterations such as changes in antennal lobe interneuron response profiles or the central nervous system olfactory integration of the incoming ORN activity.

Nevertheless, profound shifts in pheromone behavioral response profiles have been shown to have occurred in other species such as the E and Z strains of *O. nubilalis* due to alterations of the ORNs alone (Kárpáti et al. 2008). In that case, behavioral blend preferences are correlated with shifts in the primary afferents' tuning profiles, with

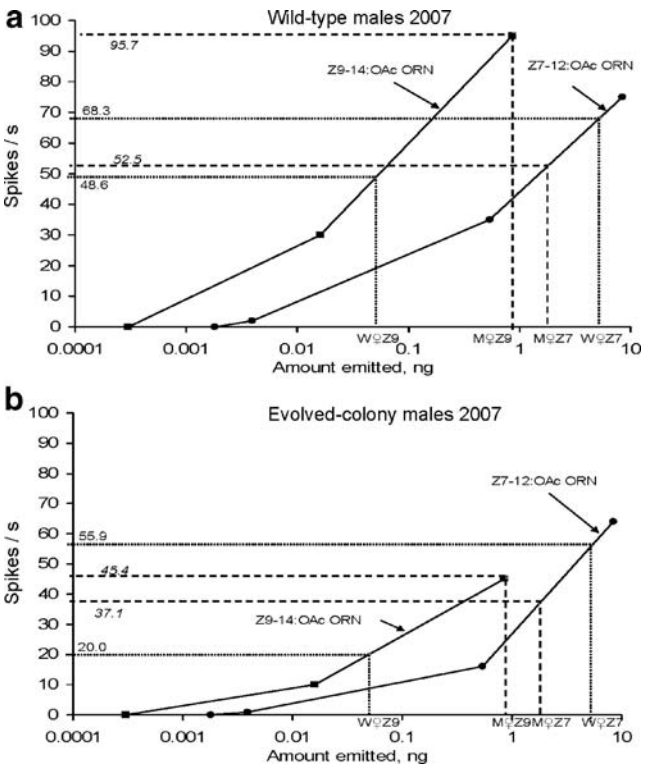

Fig. 6 Dose–response curves for the Z7-12:OAc- and Z9-14:OAc-tuned ORNs in **a** wild-type and **b** evolved-colony males using the amounts emitted from the odor cartridges. Interpolations were made to construct the firing frequencies of the ORNs in response to the amounts of Z7-12:OAc and Z9-14:OAc emitted by wild-type and mutant females in their blends (Haynes and Hunt 1990). The interpolated ratios of Z7-12:OAc/Z9-14:OAc ORNs are shown in Table 5. *Dashed line* shows mutant female emission rate. *Dotted line* shows wild-type emission rate

Table 5 Z7-12:OAc/Z9-14:OAc ORN spike frequency ratios and flight performance

2007[a]			
Wild-type males			
Wild-type female blend	69:49 spikes per second	(1.41:1)	Good flight
Mutant female blend	53:96 spikes per second	(0.55:1)	Poor flight
Evolved-colony males			
Wild-type female blend	56:20 spikes per second	(2.80:1)	Adequate flight
Mutant female blend	38:46 spikes per second	(0.83:1)	Adequate flight
1996			
Wild-type males			
Wild-type female blend	72:64 spikes per second	(1.13:1)	Good flight
Mutant female blend	54:109 spikes per second	(0.49:1)	Poor flight
Evolved-colony males			
Wild-type female blend	56:20 spikes per second	(2.80:1)	Adequate flight
Mutant female blend	39:37 spikes per second	(1.05:1)	Adequate flight

[a] See Fig. 6 for derivation of ratios

no changes in glomerular wiring (Kárpáti et al. 2008). Furthermore, the ORN alteration documented here for *T. ni* is similar in many respects with others observed that involve unusual behavioral variants of the corn borer species *O. nubilalis* and *O. furnacalis* that included broadened behavioral receptivity, characterized by upwind flight to conspecific and heterospecific pheromone blends. These rare behavioral phenotypes had correlated alterations to their ORNs, which were likely to impact relative ORN firing rates (Domingue et al. 2007a, b), as we observed here for *T. ni*. Together, the results of such studies support the generality of the "balanced olfactory antagonism" (Baker 2008) model for insect olfactory systems. In this model, there is said to be a set point for a behaviorally optimal balanced ratio of neuronal activity along mutually antagonistic odorant-specific olfactory pathways. The model predicts that a previously narrow range of pheromone component blend ratios can be expanded to include a wider range after alterations in olfactory activity occur, often at the ORN level. Differential changes in the sensitivities of differently tuned ORN classes may allow previously unacceptable pheromone blend ratios to evoke ratios of firing that fall within the acceptable balanced range (Baker 2008).

In both the *T. ni* and *Ostrinia* study systems, reductions rather than increases in ORN responsiveness appear to be the critical factors that promote flight to rare or mutant pheromone blends in laboratory populations. More specifically, these significant reductions in responsiveness tend to occur on ORNs associated with the minor pheromone components as described here for *T. ni* (also, *O. nubilalis* Domingue et al. 2007b) or with heterospecific behavioral antagonists (*O. furnacalis*, Domingue et al. 2007a). After such alterations have occurred, compounds that were previously antagonistic at low proportions in the pheromone blend can become acceptable and even promote flight at higher ratios.

The molecular basis for the observed reductions in ORN responsiveness in the rare *T. ni* males is speculative given the current level of knowledge of the functionality and expression of odorant receptors (ORs) and other factors influencing signal transduction in this species. Possible explanations might include, but are not limited to, changes to the conformations of ORs or reductions in the numbers of normal ORs expressed on the dendrites. Interestingly, if it were not for the severe decline in responsiveness of the Z9-14:OAc-tuned ORNs, the evolved males' slight reduction in responsiveness of the Z7-12:OAc-tuned ORNs alone may have actually created a less optimal ORN firing ratio for upwind flight to the mutant blend.

As described above, evolved-colony *T. ni* males are equally likely to fly upwind to mutant or wild-type blends, but respond positively to the wild-type blend less often than wild-type males do (Hemmann et al. 2008). It has been previously determined that this particular shift in behavioral response patterns can cause assortative mating between members of the two strains under certain rearing conditions (Zhu et al. 1997). It is thus possible that the physiological alteration described here represents a neuroethological cause of reproductive isolation, which involves a tradeoff between the breadth and sensitivity of the pheromone response.

Formidable challenges remain for explaining how the final stages of a speciation event could occur after the initial stages of reproductive isolation evolve because of an olfactory alteration of the particular types uncovered in the laboratories. How could the novel broadly tuned pheromone response system characteristic of the intermediate behavioral state further evolve into another highly sensitive and narrowly tuned system typical of most closely related species or races found in nature (Kárpáti et al. 2008), where reproductive isolation from the parent population is strong? Perhaps certain saltational events, such as radical changes in OR conformation, the

substitution of different ORs on dendrites, or reorganization of aspects of the central nervous system will be more likely to be adaptive when most males of a population are already in a broadly tuned state similar to that currently in existence for the evolved *T. ni* colony. After such a saltational event, it may become possible for selective pressures again to allow increases in the specificity and sensitivity of the pheromone system in a manner that continues to facilitate reproductive isolation from the parent population.

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Mechanism and Behavioral Context of Male Sex Pheromone Release in *Nasonia vitripennis*

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Abstract Males of the parasitic wasp *Nasonia vitripennis* (Hymenoptera: Pteromalidae) attract virgin females by releasing a sex pheromone composed of (4*R*,5*R*)- and (4*R*,5*S*)-5-hydroxy-4-decanolide (HDL). The pheromone is biosynthesized in the rectal vesicle of males. In the present study, we investigated the mechanism and behavioral context of pheromone release, and determined the range of activity and the longevity of the chemical signal. Our data show that the sex pheromone of *N. vitripennis* is substrate-borne and is deposited on surfaces by dabbing movements of the abdominal tip, a behavior previously described in *N. vitripennis* males as 'abdomen dipping'. Chemical markings deposited by a single male were highly attractive to virgin females. Chemical analyses revealed the presence of HDL in surface washings of marked areas, and HDL amounts correlated with male marking activity. Pheromone deposition occurred spontaneously without any additional cues being present, but marking intensity increased greatly after copulation or after a single contact with a virgin female. In contrast, marking intensity was not influenced by the presence of host puparia. Male pheromone deposits were perceived by females in a still-air olfactometer at distances of up to 4.5 cm and remained attractive for at least 2 h. The function of the substrate-borne sex pheromone is discussed with respect to the mating system of *N. vitripennis*.

Keywords Sexual communication · Abdomen dipping · Substrate-borne · Mate finding · Pteromalidae · Parasitic wasp

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Introduction

Parasitic Hymenoptera play an important regulative role in agricultural and natural ecosystems (Godfray and Cook 1997; Keeling et al. 2004). Interest in pheromone chemistry of this large and biologically diverse group is increasing, particularly because of the practical value of using natural enemies of insect pests. Sexual communication in parasitic wasps has long been assumed to be mediated mainly by female-derived pheromones that function either in attraction of males over long distances or in male courtship behavior at close range (Quicke 1997; Kainoh 1999; Ayasse et al. 2001; Steiner et al. 2005, 2006). Recent studies have revealed that, depending on the mating system, males also can be the sex that releases pheromones, thus enabling mate attraction and arrestment, respectively (Consoli et al. 2002; Ruther et al. 2007, 2008).

Nasonia vitripennis (Walker) (Hymneoptera: Pteromalidae) is a cosmopolitan ectoparasitoid that develops gregariously in pupae of cyclorrhaphous flies (Whiting 1967). The species occurs naturally in nests of hole-breeding birds and carcasses and has been a model organism for the study of parasitic wasp biology for more than four decades (Beukeboom and Desplan 2003; Shuker et al. 2003). Recent studies have demonstrated the existence of a male-derived sex pheromone in *N. vitripennis* that is highly attractive to virgin but not mated females. It is composed of a mixture of (4*R*,5*R*)- and (4*R*,5*S*)-5-hydroxy-4-decanolides (HDL) (Ruther et al. 2007). Attractiveness of HDL is synergized by the trace component 4-methylquinazoline (4-MeQ), which co-occurs with HDL in the male abdomen but is not attractive as a single compound (Ruther et al. 2008). Histological and selective chemical analyses of different abdominal tissues have revealed that HDL is synthesized in the male rectal papillae and is secreted to the

rectal vesicle (Abdel-latief et al. 2008). However, the mode and behavioral context of pheromone release, as well as the range of activity and longevity of the chemical signal, is not well understood. Previous studies on *N. vitripennis* reported that males display dabbing movements of their abdominal tips, thereby depositing fluid drops as continuous streaks (Barras 1969; van den Assem et al. 1980). This behavior, termed "abdomen dipping", is shown by males particularly after mating. Since *N. vitripennis* males are territorial, abdomen dipping has been suggested as serving as a chemical marking of male residence areas (van den Assem et al. 1980). However, these observations are anecdotal, and the exact function of the behavior remains unknown.

In this study, we tested the hypothesis that *N. vitripennis* males show abdomen dipping and deposit the sex pheromone, and we investigated the behavioral context in which this behavior is shown. For this purpose, we performed behavioral bioassays and chemical analyses to study whether: (a) male markings are attractive to virgin females, (b) the main pheromone component HDL can be detected on marked substrates, (c) HDL amounts in surface extracts correlate with the intensity of male marking behavior, and (d) the intensity of male marking behavior is influenced by previous mating, mere contact with females, or the presence of host puparia. The influence of host puparia was tested because, in many insects, pheromone calling is elicited by oviposition substrates or volatile chemicals associated with these resources (Landolt and Phillips 1997). Finally, we narrowed the range of activity and the longevity of male markings. The results are discussed with respect to the biology and the mating system of *N. vitripennis*.

Methods and Materials

Insects and General Methods Parasitic wasps were reared on pupae of the green bottle fly *Lucilia caesar* (Diptera: Calliphoridae) at 25°C, 60% relative humidity, and a photoperiod of 16:8 h (L/D) (for details, see Ruther et al. 2007). To obtain virgin parasitoids of both sexes, pupae were excised from host puparia 1–2 days before eclosion and kept individually in 1.5 ml microcentrifuge tubes (Brand GmbH, Wertheim, Germany) until emergence. The females that were used to test the attractiveness of male deposits were virgin and between 2 and 3 days old. The males that were used to investigate characteristics of the marking behavior were 2 days old and of comparable size. Size was determined by measuring the head width of fully melanised pupae under a stereo microscope with the help of a measuring eyepiece. Only pupae with a medium head width (660 to 700 μm) were used for the experiments. Mated males were obtained by keeping a single virgin female together with a virgin male in a microcentrifuge tube until mating was observed. One hour before testing, parasitoids were placed into the bioassay room to allow acclimatization to experimental conditions. Frequency of male abdomen dipping was observed under a stereomicroscope. Behavioral parameters in all experiments were recorded with the computer software The Observer 3.0 (Noldus Information Technology, Wageningen, The Netherlands). All experiments were conducted at room temperature (20–22°C). Parasitoids were used only once.

Experiment 1: Attractiveness of Male Markings Attractiveness of male markings to virgin females was examined by using a static four-cavity olfactometer. This was made of acrylic glass and consisted of a round walking arena (9-cm diameter, 8-mm thick) equipped with four symmetrically arranged spherical cavities (1-cm diameter, 4-mm deep), a low rim (2-mm high), and a glass plate to cover the arena. Single males were mated, transferred to one of the cavities (test cavity), and allowed to deposit chemical markings on the surface for 3 min. Males that did not show the characteristic abdomen dipping movements during this time were excluded from the experiment. The other three cavities remained empty and were used as controls. After males were removed, virgin females were released individually into the center of the four-cavity olfactometer, and the time they spent in test and control cavities was recorded for 5 min (N=20).

Experiment 2: Chemical Analyses of Marked Substrates - Single males of equal size were placed into a bioassay arena (1-cm diameter, 4-mm high) immediately after a copulation, and the frequency of abdomen dipping was recorded for 3 min. Subsequently, males were removed, and the surface of the arena was rinsed with 50 μl dichloromethane containing 5 ng/μl methyl undecanoate (Sigma-Aldrich, Deisenhofen, Germany) as an internal standard. The bioassay arena was made of polytetrafluoroethylene (Teflon) to minimize contamination of the extracts. Aliquots (1 μl) were analyzed by coupled gas chromatography–mass spectrometry (GC-MS) on a Fisons 8060 GC (Fisons Instruments, Mainz-Kastel, Germany) equipped with a 30 m×0.32 mm DB-5 ms capillary column (film thickness, 0.25 μm; J & W Scientific, Folsom, CA, USA) and linked to a Fisons MD800 quadrupole MS running in the electron impact (EI) mode at 70·eV. Helium (1 ml/min) was used as carrier gas. Initial oven temperature was 80°C, increased at 5°C/min to a final temperature of 280°C, and then held for 30 min. Total HDL amounts in the extracts were determined by relating peak areas to the internal standard and to the frequency of abdomen dipping displayed by the respective male (Ruther et al. 2007; N=15).

Experiment 3: Behavioral Context of Abdomen Dipping To study the behavioral context of abdomen dipping, individual males were placed into an observation chamber made of glass (3-cm diameter, 12-mm high), and the frequency of abdomen dipping was determined for 5 min under the following conditions: (a) without any additional cue (control), (b) in the presence of three unparasitized host puparia, (c) immediately after males had a single copulation with a virgin female, and (d) immediately after males had a single contact with a virgin female but were not allowed to copulate (N=15 for each condition).

Experiment 4: Longevity of Pheromone Markings To estimate the time period in which male pheromone deposits remain attractive to virgin females, we used a linear still-air olfactometer that consisted of an angular acrylic tube (14×1×1 cm) that was divided into 4-cm test and control zones at either end and with a 6-cm neutral zone in the center (Ruther et al. 2008). Microcentrifuge tubes were attached to both ends and separated from the main olfactometer arena by gauze (mesh, 0.5 mm). The tube that was connected to the test zone contained pheromone deposits released by single freshly mated males for 10 min. The tube connected to the control zone remained untreated. Volatiles were allowed to diffuse from the tubes into the olfactometer for 5 min, thus enabling the formation of an odor gradient. Subsequently, virgin females were released individually into the neutral zone, and the time parasitoids spent in test and control zone was recorded for 5 min. Odor sources were exchanged after two females had been tested. Attractiveness of the pheromone deposits was tested 0, 1, 2, and 3 h after males had deposited the pheromone (N=15). Between pheromone deposition and bioassay, microcentrifuge tubes were stored at room temperature without cover.

Experiment 5: Range of Activity of Pheromone Markings The purpose of this experiment was to estimate the distance from which *N. vitripennis* females are able to locate the pheromone source. The test was performed in a transparent polyethylene still-air Y-olfactometer (5-mm inner diameter) that consisted of a 4-cm stem and two arms of variable length (3-, 4.5-, or 6-cm distance to the branching point). Two removable polyethylene chambers (1-cm long, 4-mm inner diameter) containing pheromone deposits of a single male (test chamber, see experiment 4) or untreated (control chamber) were connected to the ends of both arms. The olfactometer was fixed vertically on a white cardboard (30×30 cm) and illuminated from above (2,050 lx). Volatiles were allowed to diffuse into the arms of the Y-tube for 5 min. Subsequently, *N. vitripennis* females were introduced individually into the olfactometer via the stem and allowed to choose one of the two arms within 3 min. Final choice was recorded when females walked at least 3 cm into one of the two arms. Females that did not make a choice within the 3 min were assumed to be unmotivated and thus were excluded from statistical analyses. Pheromone sources were renewed after every 10 min, and the olfactometer was cleaned with ethanol and demineralized water. To avoid biased results due to possible side preferences, positions of test and control chambers were exchanged regularly (N=36 for each distance).

Statistical Analyses Statistical analyses were performed by using Statistica 4.5 scientific software (StatSoft, Tulsa, OK, USA). Data in text and figures are presented as mean ± SE. The residence times that female parasitoids spent in experiment 1 in test and control cavities of the olfactometer were analyzed by a Friedman analysis of variance, followed by a Wilcoxon–Wilcox test for multiple comparisons. A Pearson correlation was done in experiment 2 to analyze the relationship between HDL amounts and the frequency of male abdomen dipping. The frequency of abdomen dipping displayed by males in experiment 3 in different situations was analyzed by a Kruskal–Wallis H test, followed by multiple Bonferroni-corrected Mann–Whitney U tests for individual comparisons. The residence time females spent in experiment 4 in the test and control zone of the linear still-air olfactometer was analyzed by a Wilcoxon matched-pairs test. Female preferences for test and control arm of the Y-olfactometer in experiment 5 were analyzed by a one-tailed binominal test.

Results

Attractiveness of Pheromone Markings (Experiment 1) Virgin females spent significantly more time in the test field that contained the male markings than in the empty control fields of the four-cavity olfactometer (Fig. 1).

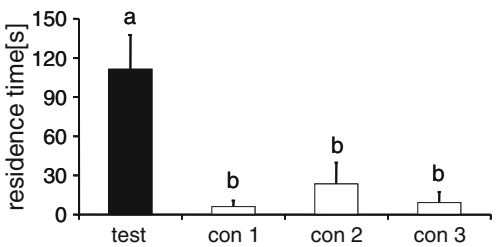

Fig. 1 Mean residence time (±SE) of virgin *Nasonia vitripennis* females in a static four-cavity olfactometer during a 5-min observation period. The test cavity contained pheromone markings deposited by a mated male for 3 min; the control cavities (con 1–3) remained untreated. *Bars with different lower case letters* are significantly different at P<0.001 (analyzed by Friedman analysis of variance, followed by Wilcoxon–Wilcox tests for multiple comparisons; N=20)

Chemical Analyses of Marked Substrates (Experiment 2) Following copulation, 80% of *N. vitripennis* males displayed abdomen dipping. The main pheromone components (4*R*,5*R*)- and (4*R*,5*S*)-HDL were detected in dichloromethane extracts of the marked substrates. During the 3-min observation period, they dipped on average 13.5±2.8 times on the substrate, thereby depositing 23.3± 5.3 ng HDL. There was a positive correlation between the frequency of abdomen dipping and the amount of total HDL determined in the surface extracts (r=0.790; t=4.649; P<0.001; N=15; Fig. 2).

Behavioral Context of Abdomen Dipping (Experiment 3) Males showed the marking behavior in all situations tested, albeit not with the same intensity (Fig. 3). They performed abdomen dipping at a very low level when additional cues were missing (5.9±2.4) or in the presence of host puparia (6.0±2.1). However, the frequency of abdomen dipping increased greatly after copulation (36.2±3.6) and even after a single contact with a virgin female (25.7±4.3).

Longevity of Pheromone Markings (Experiment 4) Females were strongly attracted to male pheromone markings 0, 1, and 2 h after pheromone deposition, and they spent significantly more time in the test zone than in the control zone of the linear olfactometer (Fig. 4). Three hours after pheromone deposition, however, females no longer responded to the markings (P=0.14).

Range of Activity of Pheromone Markings (Experiment 5) Females preferred the test arm over the control arm of the Y-olfactometer when the pheromone source was presented at distances of 3 and 4.5 cm from branching point (Fig. 5). At a distance of 6 cm, however, they did not distinguish between test and control arm (P=0.12).

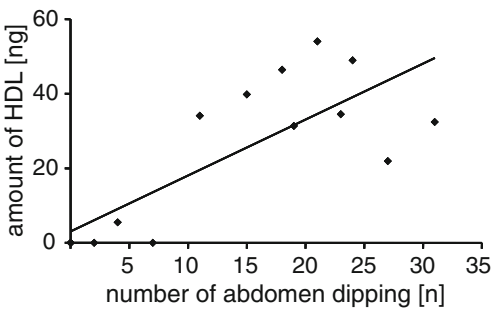

Fig. 2 Relationship between the frequency of abdomen dipping performed by *Nasonia vitripennis* males immediately after copulation during a 3-min observation period and the total amount of HDL extracted from the marked substrate (analyzed by a Pearson correlation; N=15)

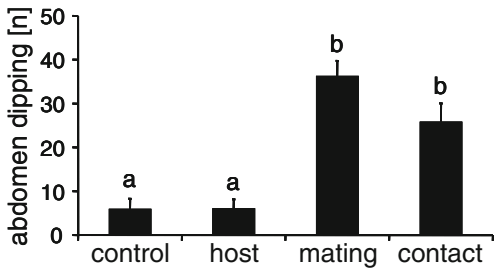

Fig. 3 Frequency of abdomen dipping by *Nasonia vitripennis* males under the following conditions: (1) without any additional cue (control), (2) in presence of host puparia, (3) immediately after mating, and (4) immediately after contact with a virgin female. *Bars with different lower case letters* are significantly different at P<0.05 (analyzed by a Kruskal–Wallis H test, followed by multiple Bonferroni-corrected Mann–Whitney U tests for multiple comparisons; each N=15)

Discussion

The study demonstrates that *N. vitripennis* males deposit their sex pheromone by the typical abdomen dipping behavior. Areas marked by a single male were strongly attractive to virgin females in the olfactometer bioassays, and GC-MS analyses revealed the presence of the major pheromone components (4*R*,5*R*)- and (4*R*,5*S*)-HDL in surface extracts. Total HDL amounts determined in these extracts correlated with the frequency of abdomen dipping displayed by individual males. The synergizing trace component 4-MeQ was not detectable in the surface washings by the applied GC-MS protocol, but the presence of this extremely odorous compound (Ruther et al. 2008) was indicated unambiguously by the characteristic medicinal odor of the marked substrates.

Pheromone production or sequestration in the rectum and release via the anal orifice is not unparalleled in

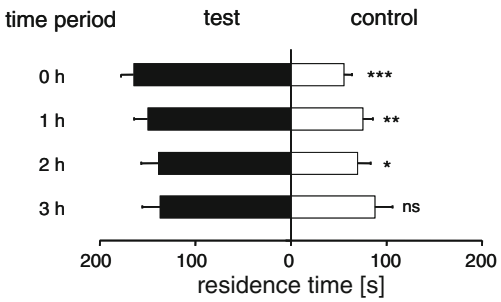

Fig. 4 Mean residence time (±SE) of virgin *Nasonia vitripennis* females in the test and control zone of a linear still-air olfactometer 0, 1, 2, and 3 h after pheromone deposition. Tubes connected to the test zone contained pheromone markings deposited for 10 min by a single male immediately after copulation; tubes of the control zone were untreated. *Asterisks* indicate significant differences (*P<0.05, **P< 0.01, ***P<0.001); *ns* not significant (analyzed by Wilcoxon matched-pairs test; each N=15)

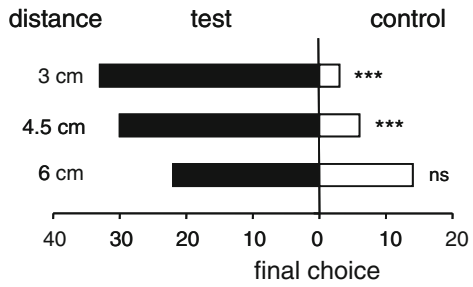

Fig. 5 Response of virgin *Nasonia vitripennis* females in a still-air Y-olfactometer to male pheromone deposits presented at different distances (3, 4.5, and 6 cm). The test arm of the Y-tube contained pheromone markings deposited for 10 min by a single male immediately after copulation; the control arm was untreated. *Asterisks* indicate significant differences (***$P<0.001$); *ns* not significant (analyzed by one-tailed binominal test; $N=36$)

insects. Males of many tephritid fruit flies use rectal pheromones to attract females (Fletcher and Kitching 1995; Khoo and Tan 2005). However, pheromone release in these insects is airborne and often supported by morphological adaptations. The melon fly *Dacus cucurbitae*, for instance, has evolved a spray mechanism by which pheromone droplets are transferred from the anus to the wings by the hind legs and subsequently are spread by wing vibration (Kuba and Sokei 1988). Substrate-borne release of rectal pheromones for recruitment purposes is known from many ant species of the taxon Formicinae (Übler et al. 1995). As in *Nasonia*, the bioactive chemicals are released via the anus by dipping movements of the abdomen (Hölldobler and Wilson 1990).

Other aculeate hymenopterans also use substrate-borne pheromones in the context of sexual communication. In bumblebees, for example, males mark spots along their patrol routes with labial gland secretions that are attractive to females (Ayasse et al. 2001). Males of the European beewolf, *Philanthus triangulum* F. (Hymenoptera, Crabronidae), attract females by marking territories with a sex pheromone secreted by the postpharyngeal gland (Kroiss et al. 2006). The use of substrate-borne sex pheromones also is documented for parasitic Hymenoptera. Females of the solitary parasitoid *Aphelinus asychis* Walker deposit a pheromone on the ground, and males follow the trail to the source by intensive antennal searching on the marked areas. However, the chemical identity and the production site of the pheromone is unknown (Fauvergue et al. 1995). A similar mate finding mechanism also has been suggested for *Ascogaster reticulatus* Watanabe (Kamano et al. 1989), *Trichogramma brassicae* Bezdenko (Pompanon et al. 1997), and *Aphytis melinus* DeBach (Bernal and Luck 2007). In contrast to the present study, however, substrate-borne sex pheromones in these parasitoids were female-derived.

The release of sex pheromones by male insects is sometimes associated with the presence of resources crucial for female reproduction (Wyatt 2003). Males of the papaya fruit fly, *Toxotrypana curvicauda*, for example, display distinct pheromone calling on the fruits used by females for oviposition (Landolt et al. 1992). In this way, responding females are rewarded with both copulation and oviposition substrate at the same site. Our results do not support such a mechanism in *N. vitripennis*, since males did not enhance the frequency of abdomen dipping in the presence of host puparia. A decisive parameter for the release of marking behavior, however, is the presence of females. Although males also showed marking behavior spontaneously and in the absence of additional cues, they clearly increased their marking activities shortly after copulation and even after mere contact with a virgin female. This suggests that males have to economize the pheromone and release higher amounts particularly when the chance is high that pheromone calling will result in mating. The mating system of *N. vitripennis* is characterized by local mate competition (Werren 1983; Shuker et al. 2005). Flightless males that emerge before females will typically mate with those emerging in rapid succession from the same host or host patch (van den Assem 1986). Hence, the presence of one virgin female indicates that others are in the vicinity, and pheromone release is likely to pay off.

Our results on the range of activity and the longevity of the chemical signal are in agreement with the *N. vitripennis* mating system. The data show that male markings attract virgin females only over relatively short distances. Pheromone molecules reach the females only by diffusion, and dispersion by wind plays a negligible role (which can be assumed in natural habitats like nests of hole-breeding birds). Thus, the male-derived sex pheromone has a range of activity of approximately 5 cm and is unlikely to assist in mate finding over longer distances. Since mating in *N. vitripennis* occurs exclusively at the site of emergence, chemically mediated long range attraction, as known particularly from solitary parasitoids (Godfray 1994), should be of no importance in this species. Male pheromone markings remained attractive for at least 2 h. Therefore, to avoid waste of resources and to make the pheromone markings a reliable signal, males should be faithful to the site of pheromone release. Indeed, it has been reported that *N. vitripennis* males sometimes stay for days at the site they have marked after copulation (van den Assem et al. 1980). However, in a previous study, males were not arrested by (4*R*,5*R*)- and (4*R*,5*S*)-HDL, the main components of their sex pheromone (Ruther et al. 2007). This suggests that other components of the abdominal secretion are involved.

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Male-Produced Aggregation Pheromone of the Lesser Mealworm Beetle, *Alphitobius diaperinus*

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Abstract The lesser mealworm beetle, *Alphitobius diaperinus* (Panzer), is a widespread serious pest in poultry production facilities and is difficult to control by conventional means. Although pheromone-based tools have become useful in the management of other beetle pests, no pheromone was known for *A. diaperinus*, and this study sought to develop basic pheromone information. Volatiles were collected in the laboratory from groups of male and female *A. diaperinus* maintained on poultry food (chick starter mash). Gas chromatographic–mass spectrometric analysis of volatiles collected from feeding males and females revealed five male-specific compounds that were identified as (*R*)-(+)-limonene, (*E*)-β-ocimene, (*S*)-(+)-linalool, (*R*)-(+)-daucene, and 2-nonanone. Emission of these began 1–2 weeks after adult emergence and could continue for at least 1 year, ceasing and resuming in response to changes in food availability and quality and other factors. No female-specific compounds were discovered. A synthetic blend of the five male compounds was attractive to both sexes in poultry production facilities in Illinois and Arkansas, indicating that the blend functions as an aggregation pheromone, but it is not yet known whether all five compounds are required for activity. A new pitfall trap is described for field use.

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Introduction

The lesser mealworm, *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae), also known as the darkling beetle or litter beetle, is a small (5–6 mm), shiny, black beetle species with a cosmopolitan distribution. This species has become an important insect pest of commercially raised poultry. Both adults and larvae are scavengers (Leschen and Steelman 1988), consuming chicken feed, feces, and dead birds and sometimes attacking living birds (Legner and Olton 1970; Pfeiffer and Axtell 1980). This insect harbors numerous avian pathogens (De las Casas et al. 1972, 1976) including *Salmonella typhimurium* (De las Casas et al. 1968; McAllister et al. 1994), *Escherichia coli* (McAllister et al. 1996), infectious bursal disease virus (McAllister et al. 1995), and *Campylobacter* (Strother et al. 2005). In addition, larvae and newly emerged adults damage poultry facility insulation as they tunnel to and from pupation sites, leading to increased heating and cooling costs (Ichinose et al. 1980; Safrit and Axtell 1984). Finally, attraction of dispersing beetles to outdoor lighting at night has led to heavy infestations in homes and businesses and, sometimes, to litigation (Gall 1980).

The lesser mealworm is difficult to control. Long-term dependence upon organic insecticides has resulted in the development of resistance. Loss of insecticide efficacy has been reported at poultry production facilities in many countries, including the USA (Wakefield and Cogan 1991;

Cogan et al. 1996; Lambkin 2005; Lambkin and Rice 2006; Steelman 2009). There is little current effort to develop new insecticides for the lesser mealworm.

Pheromones and other semiochemicals may offer alternative pest management strategies, but basic information on the chemical ecology of *A. diaperinus* is essentially lacking. There has been considerable research on semiochemicals in other Tenebrionidae, however. Defensive compounds have been described in over 100 tenebrionid species and consist primarily of quinones and 1-alkenes (Tschinkel 1975a). Cuticular hydrocarbons have been studied in tenebrionids (e.g., Lockey 1984 and references therein); these non-volatile compounds tend to occur in species-specific mixtures and likely have close-range behavioral importance. Volatile pheromones are also known. Six *Tribolium* species, some of which are serious grain pests, have male-produced aggregation pheromones (reviewed by Plarre and Vanderwel 1999), and the aldehyde, (4*R*,8*R*)-4,8-dimethyldecanal, is a major component (Suzuki and Mori 1983). One of these species, *Tribolium castaneum* (Herbst), was also found to have a female-produced pheromone, (*Z*)-2-nonenyl propionate, to which only males respond (Rangaswamy and Sasikala 1991). *Tenebrio molitor* L. has both a female-specific compound, (*R*)-4-methyl-1-nonanol (Tanaka et al. 1986), and a male-specific compound, (*Z*)-3-dodecenyl acetate (Bryning et al. 2005); both of these affect the behavior of the opposite sex. The broad-horned flour beetle, *Gnatocerus cornutus* (F.), has a male-produced aggregation pheromone, (1*S*,4*R*,5*R*)-(+)-α-acoradiene (Tebayashi et al. 1998; Tashiro et al. 2004), but no female-produced pheromone is known in this species. In *Parastizopus transgariepinus* Koch, 1-tridecene appears to function both as a component of the defensive secretion and as a male-produced sex pheromone (Geiselhardt et al. 2008). The chemical ecology of tenebrionids is complex, both biologically and chemically, and the literature provided little concrete guidance for this project.

Quinone defensive compounds have been found in *A. diaperinus* (Tseng et al. 1971). In a doctoral thesis, Falomo (1986) presented evidence for the existence of pheromones from both males and females. Her behavioral conclusions were based on laboratory pitfall bioassays. The research proceeded to the point of chromatographic purifications and examination of extracts by gas chromatography, but no chemical identifications were made.

Our study of *A. diaperinus* chemical ecology was begun to follow up on this earlier basic research. The initial approach was to compare volatiles from feeding males and females. Male-specific compounds were detected and fully characterized, but no female-specific compounds were found. Then, by using commercially available and synthetic chemicals, trapping experiments at poultry production facilities demonstrated attractiveness of the male-specific blend.

Methods and Materials

Beetles and Rearing Adult *A. diaperinus* were obtained from a commercial turkey production facility near Delavan, Tazewell County, IL, USA. The beetles were subsequently reared at NCAUR in Peoria, IL essentially by using the method of Falomo (1986). Briefly, rearing containers had a layer of wood shavings or fine vermiculite with pieces of corrugated cardboard on top, which served as pupation sites and hiding places for adults and larvae. Food for both adults and larvae was finely ground chick starter mash ("DuMor" brand, Tractor Supply Co., Brentwood, TN, USA). Water was provided, either by moistened sponges or by water-filled 5-ml vials, which were plugged with cotton dental wick and laid on their sides in the containers. The containers were misted with water every 1–3 days. Rearing containers were kept in an incubator with a temperature of 30°C, a humidity of 80–90%, and a 16:8-h (L/D) photoperiod. Periodically, young larvae were obtained by sifting rearing materials through various screens and then were transferred to new containers. Sifting also aided the recovery of pupae and prepupal larvae. Pupae were separated by sex under a microscope by using the differences in abdominal appendages described by Barké and Davis (1967). Newly emerged males and females were placed in groups by age (age range within groups was 1 week). These beetles were used for volatile collections.

Volatile Collections Erlenmeyer flasks (250 ml, with female 24/40 ground glass joints) were used as volatile collectors. Each flask was capped with a glass inlet/outlet adapter having a male 24/40 joint (catalog no. 5175, Ace Glass, Vineland, NJ, USA). Finely ground chick starter mash (approximately 5 g) was placed on the bottom of the flask and covered with a layer of vermiculite (approximately 2 cm). Water was provided by a vial with a cotton wick, as described above. Three or four cardboard squares (2×2 cm) gave additional shelter to beetles. Conditions in the volatile collectors mimicked those for successful rearing, but wood shavings were avoided because the abundant volatiles from these made the search for beetle compounds more difficult.

Adult beetles of a single sex and similar age, usually 50 in number, were added to the flask. Filters packed with Super-Q porous polymer (Cossé et al. 2002) were attached with short pieces of Teflon™ tubing to the inlet and outlet. Air was drawn through the flasks at 250–300 ml/min by a gentle vacuum connected to the outlet filter. The inlet filter served to clean incoming air, while the volatiles emitted within the flask were trapped on the outlet filter. Volatiles were recovered by rinsing filters with methylene chloride (500 μl) at intervals of 2–4 days. Temperature in the

incubator was 27°C, humidity was approximately 50%, and photoperiod was 16:8-h L/D.

During the course of the 2-year study, 20 groups of unmated males and 16 groups of unmated females were established and monitored in this way. Overall, 458 collections were made from males and 276 from females. In addition, 32 collections were made from identically prepared flasks, but without beetles. Two groups each of males and females were monitored continuously for 12 months. Each volatile collection was analyzed by gas chromatography (GC) or coupled GC mass spectrometry (GC-MS). A large number of collections were needed to detect consistent male/female differences and to distinguish them from spurious GC peaks due to occasional microbial growth or other unintended volatile sources. Selected collections were combined for further chemical analysis.

Chemical Instrumentation An HP 5890 gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) was used for the GC–flame ionization detector (GC-FID) analyses. This instrument was equipped with a flame ionization detector, cool-on-column inlet, autosampler, and a DB-5MS column (30-m length, 0.25-mm i.d., 1.0-μm film thickness; J&W Scientific, Folsom, CA, USA) and was interfaced to an HP ChemStation data system. A 20-cm retention gap of deactivated fused silica, 0.53-mm i.d., was attached to the head of the column with a press-fit glass connector to allow injections with a standard syringe. Carrier gas was helium, with inlet pressure set to 140 kPa. Oven temperature program began at 50°C for 1 min, increased at 10°C/min to 250°C, and then remained at 250°C for 3 min. Injections were made in oven-track mode (inlet temperature was always 3°C warmer than oven temperature), and detector temperature was 250°C.

For GC-MS, a 5973 mass-selective detector was interfaced to a 6890 gas chromatograph (Agilent, Santa Clara, CA, USA). For most analyses, the column was a DB-1 (30 m, 0.25-mm i.d., 1.0-μm film thickness, J&W). Carrier gas was helium and the inlet pressure was 42 kPa. The split/splitless inlet was used in splitless mode and the temperature was 200°C. Transfer line temperature was 200°C. The usual temperature program was as above for GC. Electron impact spectra were acquired at 70 eV. The Wiley mass spectral library, with 275,000 spectra, was installed on the data system.

For analysis of the enantiomers of the semiochemicals, GC-MS was performed with a Beta Dex 325 column (30 m, 0.25-mm i.d., 0.25-μm film thickness, Supelco, Bellefonte, PA, USA). Oven temperature always started at 50°C for 1 min, followed by increase at 30°C/min to the final temperature. For limonene, linalool, and daucene, the final temperatures were 90°C, 100°C, and 110°C, respectively. In each run, we verified that the GC peaks had the expected mass spectra.

Nuclear magnetic resonance (NMR) spectra were acquired on an Avance 500-MHz instrument (Bruker, Billerica, MA, USA). Samples were dissolved in $CDCl_3$ or C_6D_6. Experiments provided ^1H, ^{13}C, DEPT-135, COSY, HSQC, HMBC, and NOESY spectra. Further details are given with results.

High-performance liquid chromatography (HPLC) was conducted by using a Waters 515 pump, a Waters R401 refractive index detector, and a 25×0.46-cm i.d. silica column (Adsorbosphere Silica 5 μm, Alltech, Deerfield, IL, USA) treated with silver nitrate as described by Heath and Sonnet (1980) for separation of unsaturated compounds. Solvent was 1% 1-hexene in hexane.

Carrot Seed Extract Whole carrot seed was obtained from a local garden store, and an extract was prepared by first pulverizing the seed (78 g) in a Waring blender and then soaking the resulting meal in hexane overnight. Filtering and solvent removal under vacuum gave an oil (8 g) from which components were purified by column chromatography.

Liquid Chromatography on Open Column Volatile collections from beetles, carrot seed extract, and various synthetic products and intermediates were purified on open columns of silica gel (for separations by polarity) or silica gel containing 25% silver nitrate (for bulk separation of unsaturated hydrocarbons). In either case, solvents were various mixtures of ethyl ether and hexane. Further details are given with results.

Hydrogenation Catalytic hydrogenation of beetle- and carrot-derived compounds was conducted over 10% palladium on carbon by bubbling hydrogen gas through a methylene chloride solution for approximately 10 min, at which time no further change was observed by GC-MS.

Chemicals The following standards (Fig. 1) were acquired from Aldrich Chemical Co., Milwaukee, WI, USA: (*R*)-(+)- and (*S*)-(−)-limonene, racemic and (*R*)-(−)-linalool, and 2-nonanone. "Ocimene" [a mixture of isomers, primarily (*E*)-β-ocimene (71%) and the (*Z*)-isomer (19%), based on our analysis] came from International Flavors and Fragrances, New York, NY, USA. Synthetic reagents were also purchased from Aldrich.

(*E*)-β-ocimene was obtained from the commercial mixture in 98% purity by column chromatography on silver-nitrated treated silica (the *E* isomer was the last compound to elute; solvent was 2.5% ether in hexane). The identity of this compound was confirmed by NMR analysis (Table 1) with assignments of ^1H and ^{13}C shifts based on

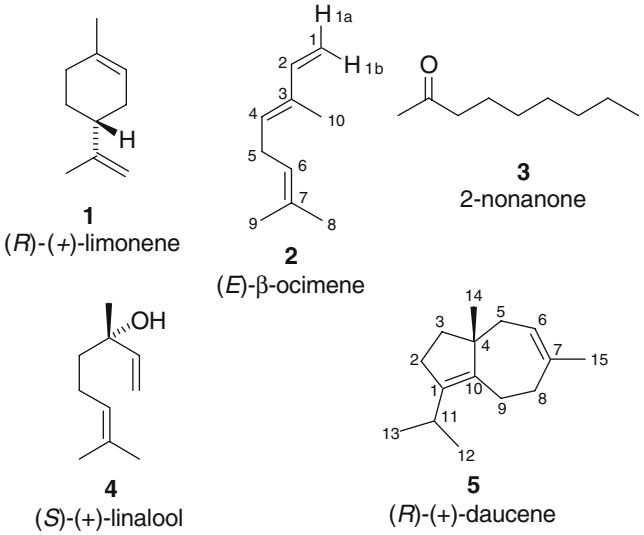

Fig. 1 Structures of key beetle-derived compounds. Position numbers for **2** and **5** correspond to Table 1 where NMR shifts are given. In (*E*)-β-Ocimene (**2**), NOESY correlations were observed between methyl 10 and olefinic proton 1b and between methyl 10 and methylene 5, but not between methyl 10 and olefinic proton 4 (the last correlation was not expected for the *E* isomer, but would occur with the *Z* isomer)

^{1}H, ^{13}C, DEPT-135, COSY, HSQC, and HMBC spectra (position numbering defined in Fig. 1). Configuration of the tri-substituted double bond between carbons 3 and 4 was confirmed to be *E* by the NOESY (nuclear Overhauser) experiment (see Fig. 1).

(*S*)-(+)-Linalool was prepared by converting the commercial (*R*)-(−)-isomer (89% e.e.) to the 4-methoxybenzoate ester, with inversion of the asymmetric center (Mukaiyama et al. 2003), followed by recovery of the alcohol by reducing the ester with diisobutylaluminum hydride (March 1985; product 79% e.e; purity by GC-FID, 98%, after column chromatography).

Racemic daucene was synthesized by the method of Naegeli and Kaiser (1972) as a standard for enantioselective GC. The initial sample of (*R*)-(+)-daucene was obtained by direct extraction from carrot seed and purification on silica gel (hexane) and $AgNO_3$-HPLC (1% 1-hexene in hexane). A richer source of (*R*)-(+)-daucene was carotol, the main constituent of carrot seed oil. This alcohol was purified on silica gel (25% ether in hexane) and dehydrated to daucene using thionyl chloride in pyridine, as described by Levisalles and Rudler (1967). After chromatography on silica gel coated with silver nitrate (elution with 2.5% ether in hexane), the (*R*)-(+)-daucene was 98% pure. No (*S*)-enantiomer was detected.

Lures for Traps Red rubber septa (11 mm, Wheaton, Millville, NJ, USA) were first cleaned by Soxhlet extraction in methylene chloride and dried. A solution of the five synthetic components (amounts as in Table 2) was then applied to each (100 μg total, in 10 μl hexane), followed by 300 μl methylene chloride, which was allowed to soak in. The two commercial components, 2-nonanone and (*R*)-(+)-limonene (>99% pure, by GC), were used as

Table 1 Assigned NMR shifts (δ) for (*E*)-β-ocimene and daucene ($CDCl_3$)

Pos	(*E*)-β-Ocimene (2)		Pos	Daucene (5)	
	^{1}H shift (description)	^{13}C shift		^{1}H shift (description)	^{13}C shift
1	H_{1a}: 4.94 (1H, br d, *J*=10.6)	110.5	1	–	138.7
	H_{1b}: 5.10 (1H, br d, *J*=17.4)		2	2.18 (2H, appar. br t, *J*=~7.5)	27.1
2	6.38 (1H, dd, *J*=10.6, 17.4)	141.5	3	1.55 (1H, m)	38.5
3	–	133.7		1.59 (1H, m)	
4	5.47 (1H, t, *J*=7.5)	131.7	4	–	49.5
5	2.84 (2H, t, *J*=7.4)	27.3	5	1.96 (1H, dd, *J*=7.8, 14)	40.4
6	5.14 (1H, br t, *J*=7.2)	122.2		2.07 (1H, br dd, *J*=5.6, 14)	
7	–	132.1	6	5.44 (1H, appar. br t, *J*=~6.6)	122.7
8	1.72 (3H, s)	25.7	7	–	139.5
9	1.66 (3H, s)	17.7	8	2.04 (2H, m)	33.5
10	1.78 (3H, s)	11.6	9	1.83 (1H, m)	22.5
				2.41 (1H, ddd, *J*=3.5, 5.1, 13.9)	
			10	–	141.9
			11	2.68 (1H, sep, *J*=6.8)	26.4
			12	0.99 (3H, d, *J*=6.8)	21.9
			13	0.94 (3H, d, *J*=6.8)	21.2
			14	0.93 (3H, s)	23.5
			15	1.76 (3H, s)	25.9

Shifts in parts per million from tetramethylsilane, coupling constants (*J*) in Hertz. Position numbers and key NOESY correlations supporting the (*E*) configuration of **2** are given in Fig. 1

Table 2 Preparation and release characteristics of lures for *A. diaperinus*

Compound	On weight basis				Relative to (*E*)-β-ocimene		
	Applied (μg)	Emission rate (ng/h)[a]		Change in emission rate per hour (%)	Applied	Emission rate (initial)	Emission rate (after 24h)
		Initial	After 24h				
(*R*)-(+)-Limonene	9.6	502 (±55)	110 (±11)	−6.5	0.16	0.16	0.16
(*E*)-β-Ocimene	60.0	3230 (±320)	665 (±60)	−6.8	1.00	1.00	1.00
2-Nonanone	3.5	174 (±16)	45 (±4)	−5.8	0.058	0.054	0.067
(*S*)-(+)-Linalool	9.6	298 (±27)	115 (±9)	−4.0	0.16	0.092	0.17
(*R*)-(+)-Daucene	17.3	173 (±10)	122 (±7)	−1.4	0.29	0.053	0.18

[a] Based on regression analysis of volatile amounts collected in laboratory, ± standard error of fitted value

received; the other three components, (*E*)-β-ocimene, (*S*)-(+)-linalool, and *R*)-(+)-daucene, were prepared as described above. Loaded septa were stored in a tightly closed bottle in the freezer until used for experiments.

Emission characteristics of three of the septa were measured in the laboratory at 27°C. Each was placed in a glass tube through which air was drawn (250 ml/min), and emitted volatiles were captured on Super-Q porous polymer (Bartelt et al. 2006). Samples were collected every 2 h for the first 8 h and then after 1 and 2 days. Amounts were determined by GC-FID and used to calculate emission rate per hour. The rate data were transformed to logarithms, and a linear regression model was fitted to describe the release behavior of each compound.

Pitfall Traps A trap was designed for field studies (Fig. 2). The trap base was a 20-cm^2, 5-mm-thick piece of plywood with a 5-cm circular hole in the center. A threaded metal canning ring (7-cm diameter; Ball Inc., Muncie, IN, USA) was attached, concentric to the hole, with three flat-head, machine screws, size 6–32 by 1.25 cm (0.5 in.), and hex nuts. Holes were predrilled, spaced equally around the canning ring. The trap top was a 10-cm^2, 5-mm-thick piece of plywood. Three holes drilled through the top aligned with the machine screws protruding through the trap base. The ends of these screws served as pegs to keep the trap top centered above the 5-cm hole, and the hex nuts served as separators between the trap top and base. Trap assembly was completed by screwing a jar into the canning ring. Jar size was either 240 ml (8 oz) or 120 ml (4 oz; Ball, Inc.).

The traps, intended to capture beetles moving about on top of poultry house litter, were set into place firmly so that the trap base was slightly below the surface of the litter. The trap tops were added to take advantage of a beetle behavior observed in the laboratory, i.e., that they often crawl into narrow spaces and beneath objects. Beetles entered the traps readily through the gap between the top and base (Fig. 2). During trap development, traps were first used without the tops, but beetles appeared reluctant to enter the center hole and trap catches were low, leading to modification into the final design.

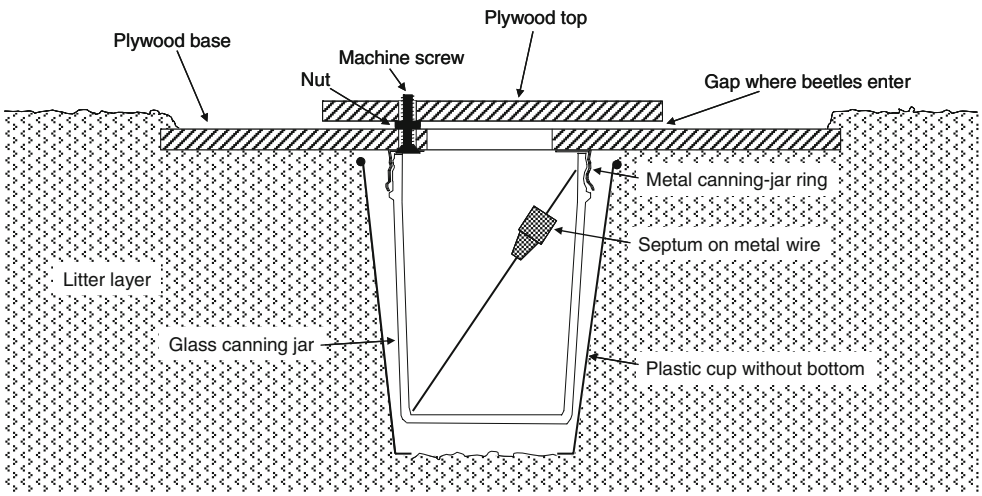

Fig. 2 Diagrammatic cross-section of trap in place during experiment. This cross-section shows one of the three screws that attach the canning ring to the trap base. See text for further description

In the Illinois field study, the wood-shaving litter was too dense for the trap simply to be twisted down into position, yet the surrounding litter quickly caved in if a hole were dug with a trowel to receive the trap jar (240-ml size). Thus, a 475-ml (16 oz) plastic cup, with the bottom removed, was first twisted down into the litter layer, with the assistance of a bulb-planting tool if resistant material was encountered. With the cup installed, the litter inside was scooped out and then the trap was seated onto the litter surface (Fig. 2). In Arkansas, the litter was loose enough so that the traps could be easily twisted into place, but the layer was relatively thin, so that the smaller 120-ml jars had to be used. Trap parts were cleaned with water and detergent and then air-dried between experiments.

Field Tests in Illinois Tests were conducted in a commercial turkey production building 10 km east of Delavan, Tazewell County, which was known to have an infestation of *A. diaperinus*. The experiments were conducted on 4 days, June 20, 24, 26, and 30, 2008, after the turkeys in the building had been moved out, but before insecticide treatment of the installation for beetles. Traps were set into the litter underneath a line of automatic feeders (for convenience, the feeders had been raised out of the way). Trap spacing was 1.5–2 m, and treatment positions within blocks (each containing two or four traps, depending on experiment) were randomized. Experiments were set out between 2:00 and 4:00 P.M. and were checked the following morning, between 8:00 and 10:00 A.M.

Experiment 1 had a paired design, and the two treatments were pheromone septa and controls. Pheromone septa were impaled on a wire (straightened paper clip) and set into the jar (Fig. 2). Thirty-four replications were acquired over the 4 days and included the nine comparisons of pheromone vs. control from experiment 2 (below). Random samples of 50 beetles from pheromone traps and 50 from controls were dissected to determine sex.

Experiment 2 had a 2^2 factorial design. The factors, pheromone, and turkey manure, were tested in the four possible combinations. A casual observation during the first 2 days of tests was that beetles aggregated around clumps of manure in the building, and this material was therefore tested as a trap treatment. Clumps of aged turkey manure were collected and, in the laboratory, were broken up and mixed thoroughly. For use in traps, the manure was wetted and 15 ml was placed in a 30-ml plastic cup, covered with fine cloth mesh secured with a rubber band to prevent entry by responding beetles. Pheromone septa were impaled on wires as above, and the cups and septa were placed in the trap jars according to the experiment plan. There were nine replications of this experiment over the final two test days.

Field Test in Arkansas The Arkansas field test was conducted August 20–23, 2008 in an infested broiler production house located 15 km west of Fayetteville, Washington County. The building was 14 m wide and 99 m long with the long axis orientated east to west and was managed to produce 6- or 8-week-old broiler chickens, but it did not contain birds at the start of the study. Two automatic feeder lines ran the length of the house, 2 m out from the wall. The floor of the house was covered with pine wood-shaving litter approximately 8 cm deep. Plastic trays containing chick feed were present; these measured 56×34 cm and were used instead of automatic feeders for newly hatched chicks during the first 10 days of a production cycle. The trays were in three parallel lines beneath each automatic feeder line, and tray spacing was 2 m, within or between lines. Beetles were abundant under the trays. The beetle traps were placed parallel to the feeder lines and between two rows of the chick feeding trays. Trap spacing was approximately 1 m. Blocks 1 and 2 were on the north side of the house and blocks 3 and 4 on the south side. Each block consisted of 12 traps, six with pheromone-treated septa and six with untreated (control) septa, which were randomly assigned within the block. Trapping was conducted over three consecutive days.

The house temperature was 28°C when the traps were initially placed and baited in the afternoon. The beetles were collected from the traps after 24 h and trap baits were replaced. Then, the house temperature was raised to 34°C (the rearing temperature of the young birds) with propane heaters. After the second 24-h period, trapped beetles were again removed and baits replaced. At this time, the chicks were released into the facility, and once again, beetles were collected from the traps after 24 h. Thus, the beetle response to the treated septa was monitored for 1 and 2 days prior to chick introduction and for 1 day afterward, and the time period of the study included a defined temperature change. Trapped beetles were transported back to the laboratory for counting.

Statistical Analysis of Field Experiment Data Trap catch data were subjected to analysis of variance after transformation by $\log(X+1)$ to stabilize variance. Linear contrasts were used in the factorial experiment to assess factor effects and their interaction. Pairwise comparison of means was by the least significant difference method ($\alpha=0.05$).

Results

Male-Specific Compounds GC and GC-MS analysis of volatile collections revealed that males feeding on chick

starter mash usually emitted five compounds that were essentially absent from females (Fig. 3). Compounds **1**–**4** were tentatively identified by a computer library search of their mass spectra (Fig. 4) as limonene, (*E*)-β-ocimene, 2-nonanone, and linalool, respectively. The identifications were confirmed by matching GC retentions and mass spectra with authentic standards, except that this analysis gave no information about the stereochemistry of **1** and **4**.

The female peak marked with a dagger in Fig. 3 had the same mass spectrum as **1** (limonene) from males, but it was later shown to be the opposite enantiomer and likely originated from the diet (see below). The compound marked with a double dagger in Fig. 3 had essentially the same GC retention as **4** (linalool), but had a different mass spectrum; this impurity was also present in many male and food-only collections and closely matched nonanal in the mass spectral library. Compound **3** (2-nonanone) was also found occasionally from females in minor amounts (i.e., <10% of the amounts in comparable male collections). It was not visible in the female sample in Fig. 3.

In contrast, no library match was found for compound **5** (mass spectrum in Fig. 4). The compound eluted from silica gel with hexane, suggesting it was not oxygenated. The apparent molecular weight (204) would correspond to the molecular formula, $C_{15}H_{24}$, likely a sesquiterpene hydrocarbon, and indicated the presence of four double-bond/ring equivalents. Serendipitously, a compound with exactly the same mass spectrum and GC retention was encountered from carrot seed oil during work on another project. Hydrogenation of both samples over palladium increased the molecular weight to 208 and generated identical GC peaks (apparently four products with nearly identical mass spectra), indicating that two double bonds were reduced and that asymmetric centers were created. The final molecular weight of 208 implied that two rings were present in **5**. An example mass spectrum of the hydrogenation products is given in Fig. 4.

The minute amount of **5** obtainable from *A. diaperinus* would not readily allow structure analysis by NMR, but the carrot seed extract supplied enough of the compound so that key NMR experiments could be run (^{1}H, COSY, HSQC, and HMBC). Deuterobenzene was used as the solvent because some sesquiterpene hydrocarbons are labile in the more commonly used, but somewhat acidic, $CDCl_3$. The compound was concluded from the spectra to be daucene (**5** in Fig. 1, but with unknown stereochemistry). Daucene was first isolated from carrots (Pigulevskii and Kovaleva 1961). Having the likely structure, we searched the literature for confirming structural and spectral information and for sources of standards for enantioselective GC. Thus, racemic daucene was synthesized and (*R*)-(+)-daucene was prepared by dehydrating carotol. Daucene from both sources was identical to the beetle and carrot compounds by non-enantioselective GC-FID and GC-MS. The literature indicated that daucene was stable in $CDCl_3$, and the NMR spectra were obtained in this solvent for the daucene sample derived from carotol for better comparison to published information. Assign-

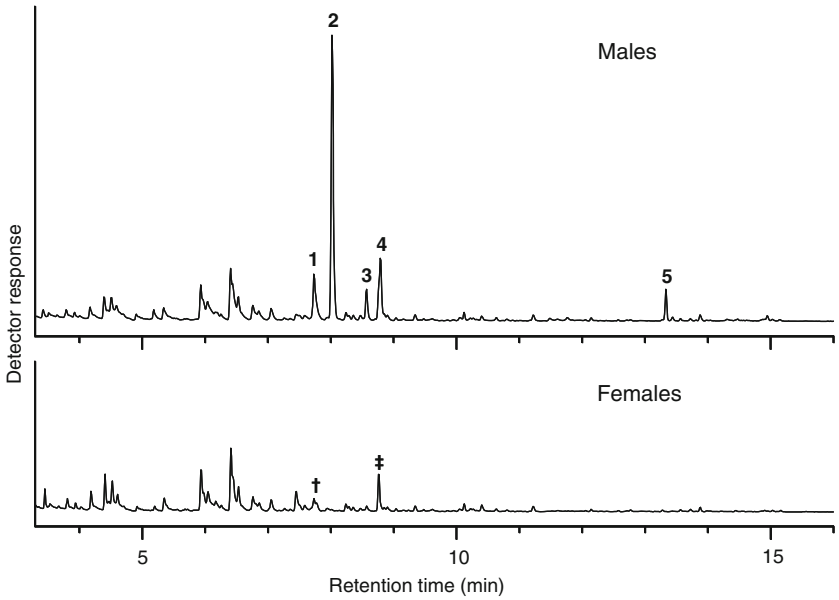

Fig. 3 Gas chromatograms of male- and female-derived volatile collections. Both chromatograms are in the same scale. Consistently encountered male-specific peaks are numbered. Peak **2** for males represents about 40 ng injected (corresponding to about 100 ng emitted per male per day in the collection flask). The peak marked with a *dagger* in the female sample has the same mass spectrum as **1**, but we concluded that it was the opposite enantiomer (see text); the peak marked with a *double dagger* has nearly the same retention time as **4**, but had an entirely different mass spectrum

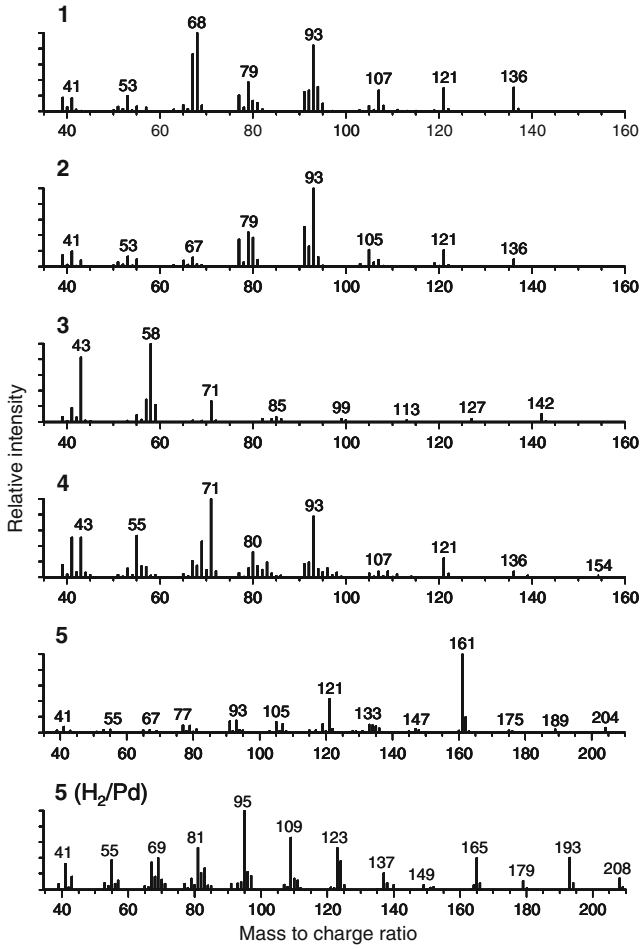

Fig. 4 Mass spectra of the five male-specific components and the hydrogenation product of **5**

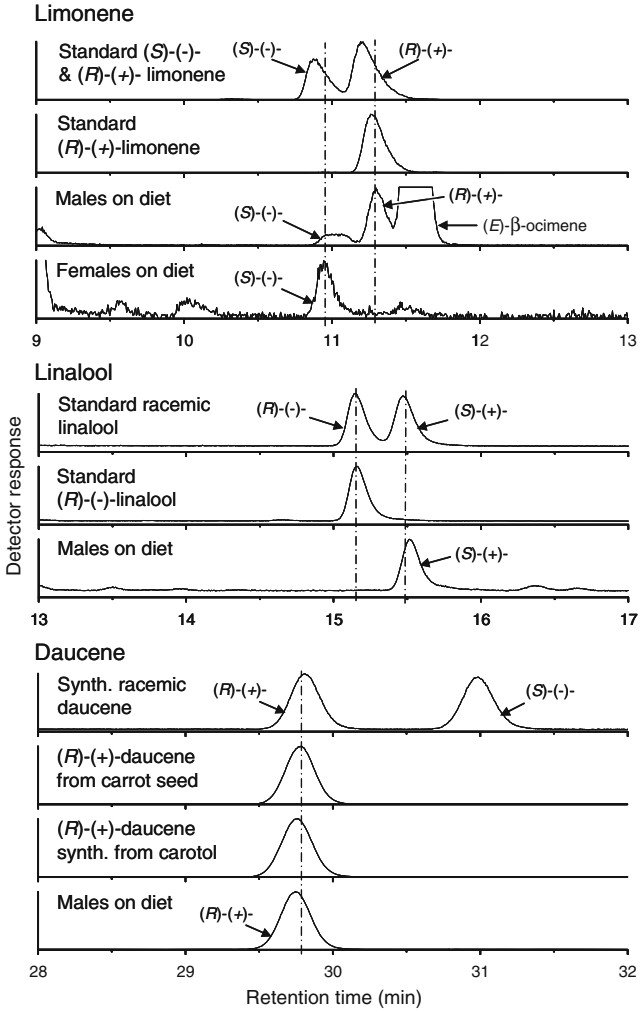

Fig. 5 Enantioselective GC analysis of beetle-derived and standard compounds

ments of resonances to structure positions are given in Table 1 (numbering as in Fig. 1). The NMR data closely matched those of Park and Little (2008), the most complete published analysis that we found.

Enantioselective GC-MS Limonene (**1**), linalool (**4**), and daucene (**5**) each have one asymmetric center, and enantioselective GC-MS was used to determine the enantiomeric composition of the natural product produced by *A. diaperinus*. The Beta-Dex 325 column was capable of separating the enantiomers of all three compounds (Fig. 5). For limonene, the (*R*)-(+)-standard was the later-eluting enantiomer, and this matched the major limonene peak from male *A. diaperinus*. However, the (*S*)-(−)-enantiomer was also detected in samples from males, females, and diet in minor amounts. We conclude that (*R*)-(+)-limonene is male-specific but that a relatively small amount of (*S*)-(−)-limonene occurs in the diet. For linalool, the commercial (*R*)-(−) standard was the earlier-eluting enantiomer, but the male-specific compound was the later-eluting one. Thus, male *A. diaperinus* emit (*S*)-(+)-linalool. The enantiomer of daucene extracted directly from carrot seed and from dehydration of carotol, reported to be (*R*)-(+)- in both cases (Levisalles and Rudler 1967; Yamasaki 1972), was the earlier GC peak, and the enantiomer from *A. diaperinus* aligned with these. Thus, males of *A. diaperinus* emit (*R*)-(+)-daucene.

Emission of Male-Specific Compounds: Biological Results Compounds **1**–**5** eventually were detected from all 20 of the monitored groups of males, but not from females [except for the opposite enantiomer of **1** (limonene) and occasional relatively minor amounts of **3** (2-nonanone), as discussed above]. Anecdotally, high production of **1**–**5** usually coincided with males walking about rapidly on the surface of the vermiculite in the collector flasks and exhibiting mating attempts toward one another. Based on 41 GC collections in which **1**–**5** stood out well above background and were easily integrated, the proportions of the compounds were fairly stable and (*E*)-β-ocimene was always the most abundant (Table 3). The compounds were

Table 3 Proportions of components in male emissions [normalized to 100 for (*E*)-β-ocimene, *N*=41]

Compound	Relative amount ± SD	Minimum, maximum
(*R*)-(+)-Limonene	16.1±2.9	11.0, 23.3
(*E*)-β-Ocimene	100	–
2-Nonanone	6.7±3.0	3.4, 21.3
(*S*)-(+)-Linalool	18.2±6.8	8.6, 40.0
(*R*)-(+)-Daucene	16.3±5.6	6.4, 28.9

A typical absolute emission rate for (*E*)-β-ocimene was 35 ng per male per day and the maximum was 120 ng per male per day

first detected 1–2 weeks after males emerged from pupae. In the groups of males monitored for 12 months, emission rate varied dramatically over time (Fig. 6). Amounts of **1**–**5** inevitably declined as food in the flask was used up or became moldy, but transferring the beetles to a clean flask with fresh food always led to a surge in the amount emitted. The conditions that reduced production of **1**–**5** did not necessarily kill the beetles, and 15 of the original 50 in the example group (Fig. 6) were still alive at the end of 1 year. Overall mean emission rate of (*E*)-β-ocimene during that period was 35±28 (SD) nanograms per day per male (*N*=108). Component proportions appeared similar to those in Table 3 throughout regardless of absolute amount.

Emission Properties of Pheromone Lures It was possible to prepare septum lures that matched the emission profile of male beetles fairly closely (Table 2), but the high volatility of the components and their considerable range of molecular weights were disadvantageous. In the laboratory evaluation, the overall emission rate decreased to 24% of the original rate after just 1 day. The proportions of components changed considerably during 1 day but generally stayed within the range measured from the beetles. Therefore, lures were used for only 1 day in field experiments and then discarded.

Field Tests in Illinois Experiment 1 demonstrated a significant treatment (pheromone) effect, and overall, the pheromone-baited traps captured 3.6 times more beetles than the controls (Table 4). Fourteen of the 68 trap catches exceeded 1,000, reflecting the large beetle population in the facility and demonstrating that beetles would enter the traps readily. Both sexes were captured in this experiment: 60 of the dissected individuals were females and 40 were males. The ratio was consistent between the pheromone treatment (31 females and 19 males) and controls (29 females and 21 males); Pearson's chi-square statistic for the two-by-two contingency table was 0.17 (1 *df*, *P*=0.68).

In experiment 2, there was also a significant treatment effect, and the partitioned effects for both the pheromone and the manure were significant, although the pheromone effect was stronger (Table 4). Given the large amount of manure that was present in the building, it seems remarkable that attraction to it could be demonstrated in the experiment. No synergism between these treatments was evident.

Field Test in Arkansas The synthetic pheromone was significantly attractive in the Arkansas experiment (Table 5).

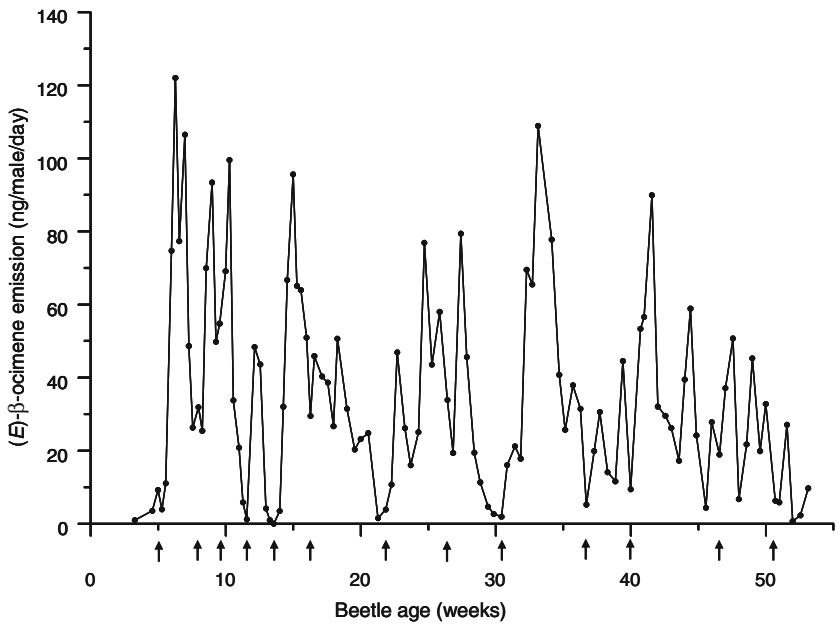

Fig. 6 Pheromone emission from one group of beetles over time, with the major component, (*E*)-β-ocimene, as the indicator. *Arrows* indicate timing of transfer to clean flasks with fresh diet

Table 4 Summary of field experiments conducted at a commercial turkey production facility near Delavan, IL, during June 20–30, 2008

Treatment	Log(X+1) scale	Untransformed
Experiment 1: Attractiveness of pheromone (paired design)		
Means (N=34)		
Control	1.80 b	61.7
Pheromone	2.35 a	222.1
Treatment effect: $F_{1,33}$=22.58 (P<0.001)		
Experiment 2: Attractiveness of pheromone and manure and combination (2×2 factorial design)		
Means (N=9)		
Control	2.4766 b	299
Pheromone	3.0248 a	1,058
Manure	2.9182 a	827
Pheromone + manure	3.1391 a	1,377
Overall treatment effect: $F_{3,24}$=8.52 (P<0.001)		
Partition of treatment effect into contrasts:		
Pheromone vs. no pheromone: $F_{1,24}$=15.0 (P<0.001)		
Manure vs. no manure: $F_{1,24}$=7.84 (P=0.01)		
Interaction: $F_{1,24}$=2.72 (P=0.11)		

Statistical analysis was on log (X+1) transformed data. Within an experiment, means followed by the same letter are not significantly different (LSD, α=0.05)

Overall, the pheromone-baited traps caught 3.3 times more beetles than controls. Captures decreased about 30-fold after the chicks were introduced (days 1 and 2 vs. day 3), for unknown reasons. Nevertheless, the factor difference between the pheromone and controls was stable among the days (treatment-by-day interaction was not significant). The statistical analysis revealed differences in total capture among the experimental blocks, indicating that

Table 5 Field experiment conducted at a broiler production facility near Fayetteville, AR, Aug 20–23, 2008[a]

Treatment	log(X+1) scale	Untransformed
Single day means (N=24)		
Day 1		
Control	2.7271 b	532
Pheromone	3.1096 a	1,287
Day 2		
Control	2.6723 b	469
Pheromone	3.2533 a	1,791
Day 3		
Control	1.1611 b	13.5
Pheromone	1.7553 a	55.9
Overall means (N=72)		
Control	2.1869 b	153
Pheromone	2.7061 a	507
ANOVA summary		
Treatment: $F_{1,129}$=33.2 (P<0.001)		
Day: $F_{2,129}$=120 (P<0.001)		
Treatment × day interaction: $F_{2,129}$=0.58 (P=0.56)		
Block: $F_{3,129}$=6.72 (P<0.001)		
Block × day interaction: $F_{6,129}$=5.23 (P<0.001)		
All interactions involving both treatment and block were not significant and were combined into residual error.		

Experiment had a randomized complete block design, with six traps for each treatment within each of four blocks, replicated on 3 days. Analysis was on log(X+1) transformed data. Within a day, or for overall means, values followed by the same letter are not significantly different (LSD, α=0.05)

the beetle population was not distributed uniformly throughout the house, and the significant interaction between blocks and days suggested that beetle distribution changed somewhat over time. Importantly, though, no interactions involving blocks and treatments were significant, indicating a consistent pheromone effect over space as well as time.

Discussion

Pheromone of A. diaperinus Based on the emission of male-specific compounds and attraction to these in traps, we conclude that *A. diaperinus* males produce an aggregation pheromone to which both sexes respond. While the blend of compounds **1**–**5** was attractive in field experiments, it is still unknown whether all five are actually required for activity. The true pheromone may be some subset of **1**–**5**, and further research is needed to address this issue. Additional research should also investigate attractants in turkey manure and other materials in the beetle habitat and test more thoroughly for synergistic interactions with **1**–**5**. It is unknown if any of the compounds **1**–**5** corresponds to the male-specific GC peak reported by Falomo (1986).

Our data suggest that the terpenoid components from *A. diaperinus* (**1**, **2**, **4**, and **5**) are synthesized by the males rather than being sequestered from food because these chemicals were not detected in the absence of males [only the opposite, (*S*)-(−)-enantiomer of limonene (**1**) was found in volatiles from females or from the beetle diet]. Traces of 2-nonanone (**3**) were sometimes detected from the food, and sequestration of this ketone by males is conceivable. However, the rather stable proportions of components in male volatiles (Table 3), despite considerable variability in absolute amounts (Fig. 6), suggest a concerted biosynthetic system that includes **3** and that keeps the ratios of all emitted volatiles fairly consistent.

Related Compounds in Other Tenebrionids None of compounds **1**–**5** was previously reported as a pheromone component in the Tenebrionidae. However, straight-chain ketones of other sizes than **3** (Tschinkel 1975b; Gnanasunderam et al. 1985) and terpenoids other than **2**, **4**, and **5** (Gnanasunderam et al. 1981; Geiselhardt et al. 2006) occur in tenebrionid defensive secretions. Limonene (**1**) has been reported in defensive secretions, but it was either the (*S*)-(−)-enantiomer (Geiselhardt et al. 2006) or its configuration was not determined (Gnanasunderam et al. 1981). In contrast to the aggregation pheromone of *A. diaperinus*, defensive secretions are released in direct response to disturbance and by both sexes. The defensive quinones of *A. diaperinus* were occasionally detected in minor amounts in volatile collections, but the emission of compounds **1**–**5** did not correlate with these, was not a result of disturbance, and was essentially sex-specific. At this point, the pheromone system of *A. diaperinus* appears most like that of *G. cornutus* (Tebayashi et al. 1998; Tashiro et al. 2004) in that only a male-produced aggregation pheromone has been detected so far and that the predominant chemicals are terpenoid.

Occurrence of **1**–**4** *in Other Insects* All of the identified compounds are well known from plants, but four of them also are known to occur in other insects and have even been reported as pheromone components. For example, (*E*)-β-ocimene and linalool (configuration not determined) are present in the hairpencil secretions of male danaid butterflies and likely have a pheromonal function (Komae et al. 1982). (*E*)-β-Ocimene occurs in the Dufour's glands of *Labidus* spp. army ants (Keegans et al. 1993). (*S*)-(+)-Linalool is part of the sex pheromone emitted by male cabbage looper moths (Heath et al. 1992). 2-Nonanone is a component of the pheromone blend emitted by female caddisflies (Löfstedt et al. 2008). Limonene and linalool are present in the defensive secretions of various true bugs (Aldrich 1988). Limonene is part of the alarm pheromone of termites (Lindström et al. 1990), is found in the mandibular glands of ants (Lloyd et al. 1989), and occurs in volatile emissions from cherry fruit flies (Raptopoulos et al. 1995). We are unaware of any previous reports of compound **5** in insects.

Practical Uses It is clear that traps in poultry buildings can capture large numbers of *A. diaperinus*, but it remains to be determined whether the pheromone can become a practical tool in managing these beetles. Trapping strategies with attractants would have to take into account factors such as the presence or absence of birds (Table 5) and the clumped but rapidly changing distribution of beetles (Strother and Steelman 2001). One positive aspect is that all five of the identified components should be readily obtainable in quantities that would allow large-scale practical use.

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Ethanol and (−)-α-Pinene: Attractant Kairomones for Bark and Ambrosia Beetles in the Southeastern US

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Abstract In 2002–2004, we examined the flight responses of 49 species of native and exotic bark and ambrosia beetles (Coleoptera: Scolytidae and Platypodidae) to traps baited with ethanol and/or (−)-α-pinene in the southeastern US. Eight field trials were conducted in mature pine stands in Alabama, Florida, Georgia, North Carolina, and South Carolina. Funnel traps baited with ethanol lures (release rate, about 0.6 g/day at 25–28°C) were attractive to ten species of ambrosia beetles (*Ambrosiodmus tachygraphus*, *Anisandrus sayi*, *Dryoxylon onoharaensum*, *Monarthrum mali*, *Xyleborinus saxesenii*, *Xyleborus affinis*, *Xyleborus ferrugineus*, *Xylosandrus compactus*, *Xylosandrus crassiusculus*, and *Xylosandrus germanus*) and two species of bark beetles (*Cryptocarenus heveae* and *Hypothenemus* sp.). Traps baited with (−)-α-pinene lures (release rate, 2–6 g/day at 25–28°C) were attractive to five bark beetle species (*Dendroctonus terebrans*, *Hylastes porculus*, *Hylastes salebrosus*, *Hylastes tenuis*, and *Ips grandicollis*) and one platypodid ambrosia beetle species (*Myoplatypus flavicornis*). Ethanol enhanced responses of some species (*Xyleborus pubescens*, *H. porculus*, *H. salebrosus*, *H. tenuis*, and *Pityophthorus cariniceps*) to traps baited with (−)-α-pinene in some locations. (−)-α-Pinene interrupted the response of some ambrosia beetle species to traps baited with ethanol, but only the response of *D. onoharaensum* was interrupted consistently at most locations. Of 23 species of ambrosia beetles captured in our field trials, nine were exotic and accounted for 70–97% of total catches of ambrosia beetles. Our results provide support for the continued use of separate traps baited with ethanol alone and ethanol with (−)-α-pinene to detect and monitor common bark and ambrosia beetles from the southeastern region of the US.

Keywords *Dendroctonus* · Ethanol · Exotic species · Host attractant · *Hylastes* · *Hypothenemus* · *Ips* · Kairomone · *Myoplatypus* · α-Pinene · Platypodidae · Scolytidae · *Xyleborinus* · *Xyleborus* · *Xylosandrus*

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Introduction

Non-native bark and ambrosia beetles (Coleoptera: Scolytidae) are regularly introduced into countries via the movement of solid wood packing materials (pallets, crating, and dunnage) used in international shipments. In New Zealand, >73% of 1,505 interception records of 103 species of bark and ambrosia beetles during 1950–2000 were associated with solid wood packing material (Brockerhoff et al. 2006). Allen and Humble (2002) reared 1,549 beetles from 29 spruce bolts (*Picea abies* L.) used as dunnage to ship granite from Norway to Canada in 1998; 95% were Scolytidae. In the US, 73% of scolytid interceptions at ports of entry from 1985 to 2000 were associated with solid wood packing material (Haack 2001).

Detection and eradication of invading species are one of three strategies that are essential in countering impacts of invasive species in the forests of the US (Chornesky et al. 2005). Direct examination of shipments for the presence of bark and ambrosia beetles is problematic, as adult and larval beetles generally are hidden within bark or wood

tissues (Allen and Humble 2002). Early detection of species that have been missed by such examinations and have attempted to escape to neighboring forest habitats is essential to successful management or eradication (Chornesky et al. 2005). One common tactic used to detect bark and wood-boring beetles employs flight traps baited with lures releasing various semiochemicals, particularly ethanol and α-pinene (Haack 2006; Liu and Dai 2006; Lee et al. 2007). In the US, national programs such as the Cooperative Agricultural Pest Survey (CAPS) and the Early Detection and Rapid Response program (EDRR) employ funnel traps baited with lures releasing ethanol or ethanol and (−)-α-pinene to capture a broad array of subcortical beetles (USDA APHIS 2007; Rabaglia et al. 2008).

In Europe, ethanol is attractive to many species of bark and ambrosia beetles such as *Anisandrus* (*Xyleborus*) *dispar* (Fabricius), *Hylastes cunicularius* Erichson, *Hylastes brunneus* Erichson, *Hylastes opacus* Erichson, *Hylurgops palliatus* (Gyllenhal), *Hylesinus* (*Leperisinus) varius* (Fabricius), *Tomicus piniperda* L., and *Trypodendron lineatum* (Olivier) (Bauer and Vité 1975; Kohnle 1985; Schroeder 1988; Schroeder and Lindelöw 1989). In British Columbia, ethanol is attractive to *T. lineatum* (Moeck 1970) and acts in synergy with the pheromone of two other species of ambrosia beetles, *Gnathotrichus sulcatus* (LeConte) and *Gnathotrichus retusus* (LeConte) (Borden et al. 1980). Kelsey and Joseph (2001) found that the bark beetle *Scolytus unispinosus* LeConte was attracted to ethanol produced by stressed branches of Douglas fir, *Pseudotsuga menziesii* (Mirb.). In the US, ethanol is attractive to *A. dispar*, *Monarthrum mali* (Fitch), *Pseudopityophthorus minutissimus* (Zimmermann), and *Xyleborinus saxesenii* (Ratzeburg) (Montgomery and Wargo 1983; Dunn and Potter 1991).

Similarly, α-pinene, a monoterpene commonly found in pine trees (Mirov 1961; Smith 2000), is attractive to various species of bark beetles such as *Hylastes nigrinus* (Mannerheim), *Ips grandicollis* (Eichhoff), *Ips typographus* L., and *T. piniperda* (Rudinsky et al. 1971; Schroeder and Eidmann 1987; Witcosky et al. 1987; Schroeder 1988; Erbilgin and Raffa 2000). The combination of ethanol and α-pinene is attractive to bark and ambrosia beetles such as *Dendroctonus valens* LeConte, *Dryocoetes autographus* (Ratzeburg), *G. retusus*, *G. sulcatus*, *H. palliatus* (Gyll.), *Hylastes porculus* Erichson, *T. lineatum*, and *T. piniperda* (Borden et al. 1981; Schroeder 1988; Chénier and Philogène 1989; Schroeder and Lindelöw 1989; Erbilgin et al. 2001).

However, data on the attractiveness of such lures to bark and ambrosia beetles are limited to a small portion of known species (<1% of nearly 6,000 species; Seybold et al. 2006). Specifically lacking are response data for species common to the southeastern US. Attraction of *I. grandicollis* to small loblolly pine logs in the southeastern US was enhanced with the application of pinenes and turpentine directly onto the bark (Anderson 1977). In the northern portion of its range (Wisconsin and southern Ontario), monoterpenes including α-pinene affected trap captures of *I. grandicollis* (Chénier and Philogène 1989; Erbilgin and Raffa 2000). In Florida, the combination of ethanol and turpentine was attractive to *Dendroctonus terebrans* (Olivier), *Hylastes salebrosus* Eichhoff, and *Xyleborus pubescens* Zimmermann, whereas *Xyleborus affinis* Wood was attracted to ethanol but not turpentine (Fatzinger 1985; Fatzinger et al. 1987; Phillips et al. 1988; Phillips 1990). The use of turpentine in these studies is a concern, as the monoterpene composition of turpentine can vary widely depending on species and location of conifers used in production (Mirov 1961; Smith 2000). The main constituent of the turpentine used in at least one of the Florida studies was α-pinene (Phillips et al. 1988).

A second concern is that α-pinene may interrupt attraction of some ambrosia beetles to ethanol. In southwestern British Columbia (Canada), α-pinene seems to interrupt catches of *A. dispar*, *X. saxesenii*, *Xyleborinus alni* (Niisima), *Xyleborus pfeili* (Ratzeburg), and *Xylosandrus germanus* (Blandford) (L. Humble, unpublished data). Schroeder and Lindelöw (1989) found that α-pinene interrupted catches of *A. dispar* to ethanol-baited traps in Sweden. In Europe, Lindelöw et al. (1993) found that α-pinene interrupted the attraction of *H. cunicularius*, *H. opacus*, and *D. autographus* to a blend of ethanol and turpentine. For this reason, most operational detection programs for ambrosia beetles use traps baited solely with ethanol in addition to traps baited with ethanol and α-pinene or α-pinene alone to avoid the risk of species evading detection.

Our objective was to assess the attraction of bark and ambrosia beetles (common to pine forests of the southeastern US) to standard commercially available ethanol and (−)-α-pinene lures used with funnel traps. We wanted to determine: (1) the species that are attracted to traps baited with these compounds in southern pine forests and (2) if traps baited with the combination of the two lures were as effective as, if not better than, traps baited solely with one lure. We monitored the responses of 49 species of common bark and ambrosia beetles to traps baited with ethanol and/or (−)-α-pinene in southeastern US (Tables 1 and 2). Our data on Buprestidae, Cerambycidae, Curculionidae, and Elateridae were published previously (Miller 2006). Our goal is to provide operational guidelines for the use of semiochemical-based trapping systems in monitoring populations of beetles in the southeastern US, as well as in suppression and detection programs in countries where these species may not be native.

Methods and Materials

Experimental Design Methods and materials were reported previously in Miller (2006). The same experimental design

Table 1 National forest (NF) and experimental forest (EF) locations, forest types, and trapping dates for experiments (2002–2004) in the southeastern US

Expt.	Location	Tree species	Trapping dates
1	Ocala NF near Salt Springs, FL	*P. palustris* Miller	26 February–26 May 2002
2	Osceola NF near Lake City, FL	*P. palustris* and *P. elliottii* Engelmann	25 February–25 May 2002
3	Oconee NF near Juliette, GA	*P. taeda* L.	12 June–8 August 2002
4	Blue Valley EF near Highlands, NC	*P. strobus* L.	20 June–20 August 2002
5	Bankhead NF near Grayson, AL	*P. taeda* and *T. canadensis* (L.)	28 April–10 July 2003
6	Nantahala NF near Murphy, NC	*P. strobus* and *P. echinata* Miller	1 May–14 August 2003
7	Sumter NF near Union, SC	*P. taeda*	15 April–16 July 2003
8	Apalachicola NF near Tallahassee, FL	*P. palustris* and *P. elliottii*	30 March–16 June 2004

was employed in mature pine stands on each of seven National Forests (NF) and one Experimental Forest (EF) in the southeastern US, resulting in eight experiments (Table 1). In each experiment, four treatments were randomly assigned to four eight-unit funnel traps [Phero Tech (now Contech) Inc., Delta, British Columbia, Canada] within each of eight replicate blocks (N=8) as follows: (1) unbaited control; (2) ethanol alone; (3) α-pinene alone; and (4) ethanol (+)-α-pinene. Traps were spaced 10–15 m apart within blocks; replicate blocks were spaced 15–500 m apart. Phero Tech Inc. supplied sealed ultra-high-release (UHR) plastic pouches containing either ethanol (150 ml) or α-pinene (200 ml; chemical purities >95%). The enantiomeric purity of α-pinene was >95%-(−). The release rate of ethanol from its UHR pouch was 0.6 g/day at 25–28°C (determined by weight loss). (−)-α-Pinene was released at 2–6 g/day from its UHR pouch at 25–28°C (determined by weight loss). Each trap was suspended between trees by rope such that the bottom of the trap was 0.2–0.5 m above ground level. Collection cups contained 150–200 ml of pink propylene glycol solution (Peak RV and Marine Antifreeze, Old World Industries Inc., Northbrook, IL, USA) as a killing and preservation agent. Voucher specimens were deposited in the Entomology Collection, Museum of Natural History, University of Georgia (Athens, GA, USA).

Statistical Analyses Trap catch data were analyzed with the SYSTAT (ver. 11.00.01) and the SigmaStat (ver. 3.01) statistical packages (SYSTAT Software Inc., Point Richmond, CA, USA) for locations where sufficient numbers ($N \geq 50$) were captured for individual species. Trap catch data were transformed by $\ln(Y+1)$ to remove heteroscedasticity (Pepper et al. 1997). In each experiment, trap catch data were subjected to analysis of variance (ANOVA) by using the following model components: (1) replicate; (2) ethanol; (3) α-pinene; and (4) ethanol × α-pinene. In cases with one treatment lacking variation (due to lack of any beetle captures), the data were subjected to ANOVA by using the following model components: (1) replicate and (2) treatment. In all experiments with three to four non-zero treatment means, the Holm–Sidak multiple-comparison procedure (Glantz 2005) was used to compare means within a location for each species when there was a significant treatment effect (α=0.05). In cases with two treatments lacking variation (due to lack of any beetle captures), the means of catches associated with the two remaining treatments were analyzed by two-tailed t tests.

Results

Ambrosia Beetles A total of 28,521 ambrosia beetles were captured from all eight locations in 2002–2004, ranging from 972 to 10,683 per location (Table 2). Of 23 species captured in our trapping studies, nine are established non-native exotic species and accounted for 69.7–96.6% of total ambrosia beetle catches. The most common non-native species were *X. saxesenii*, *Xylosandrus crassiusculus* (Motschulsky), and *Dryoxylon onoharaensum* (Murayama), whereas the most common native species were *X. affinis*, *Xyleborus ferrugineus* (Fabricius), and *X. pubescens*. Generally, ethanol had an attractive effect on most ambrosia beetles with (−)-α-pinene playing a minor role (Table 3).

The exotic ambrosia beetle *X. saxesenii* was captured at all eight locations with a total catch of 13,671 (accounting for 48% of total ambrosia beetle catches; Fig. 1). Ethanol had a significant effect on trap catches of *X. saxesenii* (Table 3). At all locations, traps baited with ethanol [with or without the addition of (−)-α-pinene] caught more *X. saxesenii* than unbaited control traps. At most locations, (−)-α-pinene had no effect on catches of *X. saxesenii* (Table 3). However, catches of *X. saxesenii* in traps baited with ethanol were interrupted by (−)-α-pinene in Florida (Ocala NF) and North Carolina (Nantahala NF; Fig. 1e, f).

Catches of the exotic ambrosia beetles *Xylosandrus compactus* (Eichhoff), *X. crassiusculus*, and *X. germanus*

Table 2 Total catches of ambrosia and bark beetles at eight experimental sites in the southeastern US

Species	Experiment and state							
	1 FL	2 FL	3 GA	4 NC	5 AL	6 NC	7 SC	8 FL
Ambrosia beetles								
Ambrosiodmus lecontei Hopkins	7	11						18
Ambrosiodmus obliquus (LeConte)	28	44	2		17	13	12	25
Ambrosiodmus rubricollis (Eichhoff)[a]		1	9		9		13	2
Ambrosiodmus tachygraphus Zimmermann				1	1	57		
Anisandrus (*Xyleborus*) *sayi* (Hopkins)				26	9	103		
Corthylus columbianus Hopkins		4						1
Corthylus papulans Eichhoff	2	14						
Dryoxylon onoharaensum (Murayama)[a]	199	1,883	208	48	32	167	528	3,506
Euplatypus compositus (Say)	4	1						
Euwallacea validus (Eichhoff)[a]			30		10			
Gnathotrichus materiarus (Fitch)	2		2	14		70	12	
Monarthrum fasciatum (Say)	76	12	2			2		
Monarthrum mali (Fitch)	36	9	15	50	10	15	2	2
Myoplatypus flavicornis (Fabricius)	2	5			28	20	65	
Premnobius cavipennis Eichhoff[a]	1							
Xyleborinus saxesenii (Ratzeburg)[a]	1,020	3,297	328	2,492	417	994	889	4,234
Xyleborus affinis Eichhoff	57	359	15	3			6	187
Xyleborus atratus Eichhoff[a]		3	3	15	16	34	2	14
Xyleborus ferrugineus (Fabricius)	17	110	52	93	122	9	12	135
Xyleborus pubescens Zimmermann	178	645	3	4	24	333	187	
Xylosandrus compactus (Eichhoff)[a]	4	5	4		43	1		344
Xylosandrus crassiusculus (Motschulsky)[a]	7	125	298		1,286	212	7	2,215
Xylosandrus germanus (Blandford)[a]			1	149	3	21		
Total	1,640	6,528	972	2,895	2,017	2,051	1,735	10,683
Bark beetles								
Carphoborus bicornis Wood	1				1			
Cnesinus strigicollis LeConte	2	2	26	3		2		5
Conophthorus coniperda (Schwarz)				1				
Cryptocarenus heveae (Hagedorn)	282		1					
Crypturgus alutaceus Schwarz	1						1	
Dendroctonus frontalis Zimmermann	2				4		4	
Dendroctonus terebrans (Olivier)	730	757	389	30	2	131	153	424
Hylastes porculus Erichson			225	453	185	399	157	
Hylastes salebrosus Eichhoff	196	194	71	28	208	43	907	326
Hylastes tenuis Eichhoff	4	38	274	233	75	805	773	84
Hylurgops rugipennis pinifex (Fitch)						1		
Hypothenemus Westwood sp.	16	36	23	3	81	16	9	20
Ips avulsus (Eichhoff)	4	23				1		
Ips calligraphus (Germar)							4	
Ips grandicollis (Eichhoff)	1,033	1,846	444	282	151	234	425	1,418
Micracisella nanula (LeConte)		1	1					
Orthotomicus caelatus (Eichhoff)	21	13	5	6	2	12	40	10
Phloeosinus taxodii taxodii Blackman							2	
Phloeotribus liminaris (Harris)					4			
Pityophthorus cariniceps LeConte				50				

Table 2 (continued)

Species	Experiment and state							
	1 FL	2 FL	3 GA	4 NC	5 AL	6 NC	7 SC	8 FL
Pityophthorus confusus bellus Blackman						5	4	
Pityophthorus consimilis LeConte						2		
Pityophthorus lautus Eichhoff			2			6		
Pityophthorus pulicarius (Zimmermann)	4	1						
Pseudopityophthorus pruinosus (Eichhoff)		3						
Scolytus multistriatus (Marsham)[a]			6					
Total	2,296	2,914	1,467	1,089	713	1,657	2,479	2,287
Grand total	3,936	9,442	2,439	3,984	2,730	3,708	4,214	12,970

All species are Scolytidae (Coleoptera) except *Euplatypus compositus* and *M. flavicornis*, which are in the Platypodidae

[a] Exotic species

totaled 4,725 beetles (17% of total ambrosia beetle catches) with *X. crassiusculus* being the most common (Fig. 2). Ethanol had a significant effect on trap catches of all three species (Table 3). In five locations, catches of *X. crassiusculus* in traps baited with ethanol [with or without the addition of (−)-α-pinene] were greater than those in unbaited control traps (Fig. 2a–e). The same was true for *X. compactus* in Florida (Apalachicola NF; Fig. 2f) and *X. germanus* in North Carolina (Blue Valley EF; Fig. 2g). There was no effect of (−)-α-pinene on any of these three species (Table 3, Fig. 2).

An interruptive effect of (−)-α-pinene on the attraction of ambrosia beetles to ethanol-baited traps was apparent with two native species, *Ambrosiodmus tachygraphus* Zimmermann, *Anisandrus* (*Xyleborus*) *sayi* (Hopkins), and an exotic species, *D. onoharaensum* (Table 3, Fig. 3). All three species were attracted to traps baited with ethanol. In North Carolina (Nantahala NF), catches of *A. tachygraphus* in traps baited with ethanol alone were significantly greater than those in unbaited controls, whereas catches in traps baited with ethanol and (−)-α-pinene were not different from those in control traps (Fig. 3a). No beetles were captured in traps baited solely with (−)-α-pinene. In North Carolina (Nantahala NF), catches of *A. sayi* in traps baited with ethanol and (−)-α-pinene were significantly less than those in traps baited solely with ethanol (Fig. 3b). No beetles were caught in control traps or traps baited solely with (−)-α-pinene. In five of six locations, catches of *D. onoharaensum* in traps baited with ethanol and (−)-α-pinene were significantly less than those in traps baited solely with ethanol (Fig. 3c, e–h). We caught a total of 6,571 *D. onoharaensum* across the six locations (23% of total ambrosia beetle catches; Fig. 3c–h).

Lastly, ethanol and (−)-α-pinene significantly affected catches of five common native species of ambrosia beetles (Table 3, Figs. 4 and 5), although results were inconsistent between locations. Catches of *X. affinis*, *X. pubescens*, and *X. ferrugineus* totaled 2,458 beetles (9% of total ambrosia beetle catches). Traps baited with ethanol were attractive to *X. affinis* and *X. ferrugineus* in three and five locations, respectively (Figs. 4a–c and 5a–e), whereas *X. pubescens* were attracted to ethanol-baited traps in only one of four locations (Fig. 4g). Traps baited with (−)-α-pinene alone were attractive to *X. pubescens* in Florida (Osceola NF) with (−)-α-pinene enhancing catches of beetles in ethanol-baited traps (Fig. 4f). *X. ferrugineus* were attracted to traps baited with (−)-α-pinene in two locations in Florida (Fig. 5b, c) but interrupted by (−)-α-pinene in Alabama (Fig. 5a). (−)-α-Pinene had no effect on *X. affinis* at four locations (Fig. 4a–c). *M. mali* was attracted to traps baited with ethanol (Fig. 5f), whereas the platypodid *Myoplatypus flavicornis* (Fabricius) was attracted to traps baited with (−)-α-pinene (Fig. 5g). As with the other species for which numbers were too low for analyses ($N<50$) at some locations (Table 2), the low numbers of *M. mali* or *M. flavicornis* in traps at the other locations may have been a consequence of low population numbers or a lack of attraction at these localities.

Bark Beetles A total of 14,902 bark beetles were captured from all eight locations in 2002–2004, ranging from 713 to 2,914 per location (Table 2). Of 26 species captured in our trapping studies, only *Scolytus multistriatus* (Marsham) is a non-native species. The most common bark beetles were *D. terebrans*, *H. porculus*, *H. salebrosus*, *Hylastes tenuis* Eichhoff, and *I. grandicollis*. In contrast to ambrosia beetles, (−)-α-pinene had an effect on most bark beetle species with ethanol playing a minor role (Table 4). *Cryptocarenus heveae* (Hagedorn) and a *Hypothenemus* Westwood sp. were the only two species of bark beetles that

Table 3 Significance levels for ANOVAs on effects of ethanol and (−)-α-pinene on ambrosia beetle trap catches in the southeastern US

Species	Location	Rep	Ethanol (E)	α-Pinene (A)	E×A
Dryoxylon onoharaensum	FL—Apalachicola NF	0.033	<0.001	0.001	0.070
	FL—Ocala NF	0.990	<0.001	0.012	0.023
	FL—Osceola NF	0.033	<0.001	0.106	0.063
	GA—Oconee NF	0.277	<0.001	0.171	0.171
	NC—Nantahala NF	0.093	<0.001	0.006	0.015
	SC—Sumter NF	0.984	<0.001	0.002	0.110
Gnathotrichus materiarus	NC—Nantahala NF	<0.001	0.852	0.078	0.993
Monarthrum fasciatum	FL—Ocala NF	0.115	0.384	0.384	0.354
Myoplatypus flavicornis	SC—Sumter NF	0.315	0.660	<0.001	0.233
Xyleborinus saxesenii	AL—Bankhead NF	0.021	<0.001	0.413	0.366
	FL—Apalachicola NF	0.009	<0.001	<0.001	0.006
	FL—Ocala NF	0.179	<0.001	0.299	0.002
	FL—Osceola NF	0.435	<0.001	0.528	0.291
	GA—Oconee NF	0.051	<0.001	0.171	0.287
	NC—Blue Valley EF	0.306	<0.001	0.517	0.668
	NC—Nantahala NF	0.051	<0.001	0.140	0.027
	SC—Sumter NF	0.322	<0.001	0.247	0.356
Xyleborus affinis	FL—Apalachicola NF	0.423	<0.001	0.002	0.527
	FL—Ocala NF	0.531	<0.001	0.723	0.822
	FL—Osceola NF	0.307	<0.001	0.955	0.119
Xyleborus ferrugineus	AL—Bankhead NF	0.019	<0.001	<0.001	0.554
	FL—Apalachicola NF	0.253	<0.001	0.517	0.004
	FL—Osceola NF	0.079	0.166	<0.001	0.008
	GA—Oconee NF	0.060	<0.001	0.761	0.853
	NC—Blue Valley EF	0.381	<0.001	0.177	0.310
Xyleborus pubescens	FL—Ocala NF	0.230	0.282	0.002	0.144
	FL—Osceola NF	0.083	0.005	<0.001	0.278
	NC—Nantahala NF	0.001	0.102	0.968	0.070
	SC—Sumter NF	0.064	<0.001	0.053	0.933
Xylosandrus compactus	FL—Apalachicola NF	0.901	<0.001	0.317	0.317
Xylosandrus crassiusculus	AL—Bankhead NF	0.005	<0.001	0.557	0.286
	FL—Apalachicola NF	0.354	<0.001	0.488	0.590
	FL—Osceola NF	0.034	<0.001	0.532	0.532
	GA—Oconee NF	0.659	<0.001	0.744	0.637
	NC—Nantahala NF	0.519	<0.001	0.548	0.309
Xylosandrus germanus	NC—Blue Valley EF	<0.001	<0.001	0.236	0.125

were attracted to ethanol-baited traps with no effect from (−)-α-pinene (Fig. 6a, b).

A total of 2,616 black turpentine beetles *D. terebrans* were captured across six locations (18% of total bark beetle catches; Fig. 6c–h). Catches of *D. terebrans* were significantly affected by (−)-α-pinene with little, if any, effect of ethanol (Table 4). At all locations, traps baited with (−)-α-pinene (with or without the addition of ethanol) caught more *D. terebrans* than unbaited control traps. However, in Florida (Ocala NF), catches of *D. terebrans* in traps baited with (−)-α-pinene were enhanced by the addition of ethanol (Fig. 6e); there was no effect of ethanol at the other locations (Fig. 6a–d, f).

The eastern five-spined ips, *I. grandicollis*, was captured at all eight locations with a total catch of 5,833 beetles (39% of total bark beetle catches; Fig. 7). As with *D. terebrans*, catches of *I. grandicollis* were significantly affected by (−)-α-pinene at all locations with inconsistent effects of ethanol among locations (Table 4). At all locations, traps baited with (−)-α-pinene (with or without the addition of ethanol) caught more *I. grandicollis* than blank control traps. The addition of ethanol to traps baited with (−)-α-pinene enhanced catches of *I. grandicollis* in Alabama (Fig. 7a) but interrupted catches in Florida (Ocala NF; Fig. 7e). There was no effect of ethanol at the other locations (Fig. 7b–d, f, g).

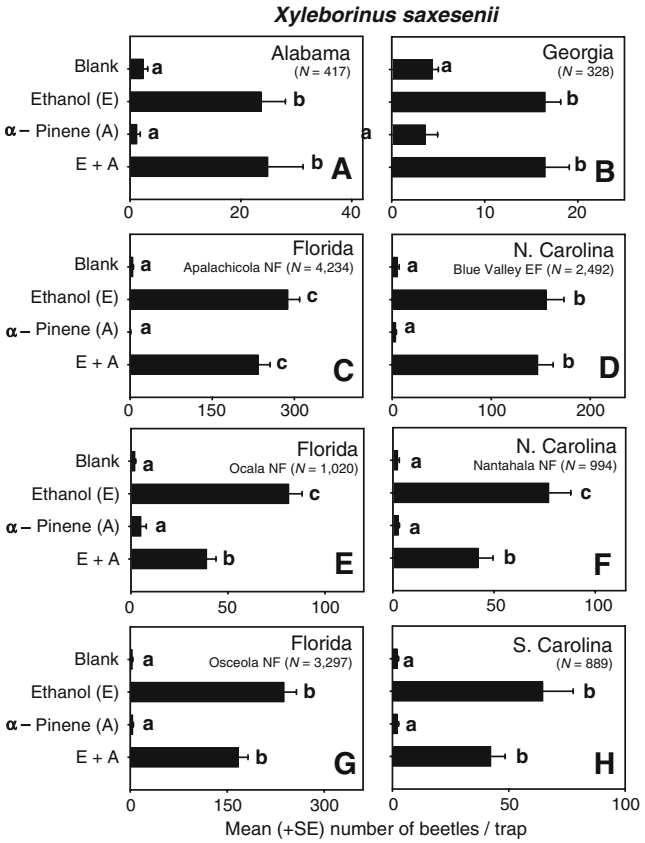

Fig. 1 **a**–**h** Effects of ethanol and (−)-α-pinene on trap catches of *X. saxesenii* (Scolytidae) in the southeastern US. Means followed by the same *letter* are not significantly different (α=0.05, Holm–Sidak multiple comparison)

Catches of *H. porculus*, *H. salebrosus*, and *H. tenuis* totaled 5,678 (38% of total bark beetle catches) with *H. tenuis* the most common (Figs. 8, 9, and 10a–f). (−)-α-Pinene had a significant effect on all three species (Table 4). At all locations, traps baited with (−)-α-pinene (with or without the addition of ethanol) caught more *H. porculus* and *H. tenuis* than blank control traps (Figs. 8 and 9). The effect of ethanol on catches of *H. porculus* and *H. tenuis* was inconsistent among locations (Table 4). Ethanol enhanced catches of *H. tenuis* to traps baited with (−)-α-pinene in Florida (Apalachicola NF; Fig. 8c) but not at the other five locations (Fig. 8a, b, d–f). Similarly, catches of *H. porculus* in traps baited with (−)-α-pinene were enhanced by the addition of ethanol in Georgia and North Carolina (Blue Valley EF; Fig. 9c, e) but not at the other three locations (Fig. 9a, b, d).

The effects of ethanol and (−)-α-pinene on catches of *H. salebrosus* were fairly consistent among six locations (Table 4). At all six locations, catches of *H. salebrosus* were highest in traps baited with both ethanol and (−)-α-pinene (Fig. 10). At four of six locations, traps baited with (−)-α-pinene caught more *H. salebrosus* than control traps (Fig. 10a–c, f). In South Carolina, traps baited with ethanol were more attractive than control traps (Fig. 10f). Similarly, catches of *Pityophthorus cariniceps* LeConte were highest in traps baited with ethanol and (−)-α-pinene (Fig. 10g).

Discussion

In addressing our first objective, we found that traps baited with ethanol and/or (−)-α-pinene were attractive to 20 species of common bark and ambrosia beetles in the southeastern US, including five exotic species of ambrosia beetles. In southern pine forests, traps baited with (−)-α-pinene were attractive to the bark beetles, *D. terebrans*, *I. grandicollis*, *H. tenuis*, *H. porculus*, and *H. salebrosus* (Figs. 6, 7, 8, 9, and 10) and the platypodid ambrosia beetle *M. flavicornis* (Fig. 5g). In some locations, traps baited with (−)-α-pinene were attractive to the ambrosia beetles, *X. ferrugineus* (Fig. 5b, c) and *X. pubescens* (Fig. 4f).

Our results with *D. terebrans* are consistent with previous tests that employed turpentine (Fatzinger et al.

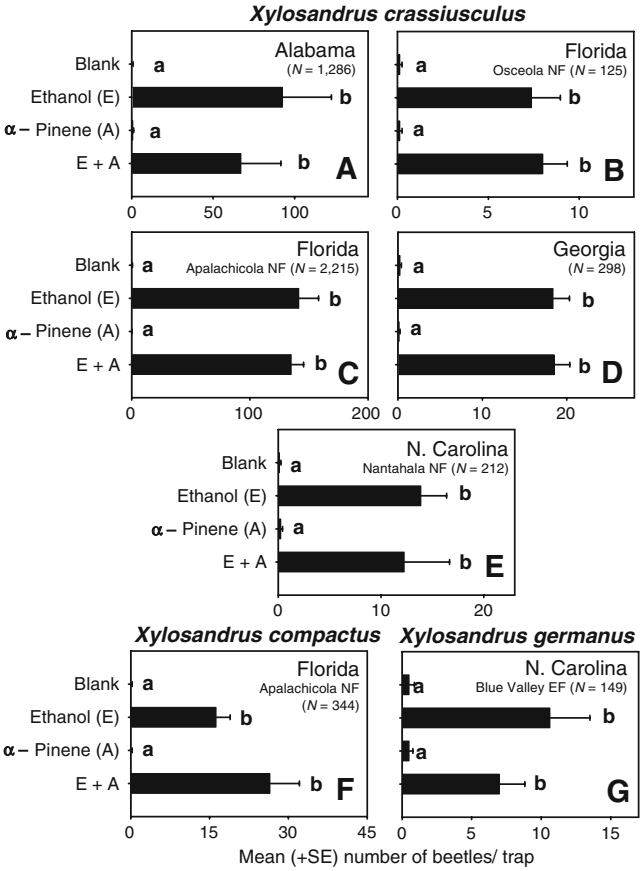

Fig. 2 Effects of ethanol and (−)-α-pinene on trap catches of *X. crassiusculus* (**a**–**e**), *X. compactus* (**f**), and *X. germanus* (**g**; Scolytidae) in the southeastern US. Means followed by the same *letter* are not significantly different (α=0.05, Holm–Sidak multiple comparison)

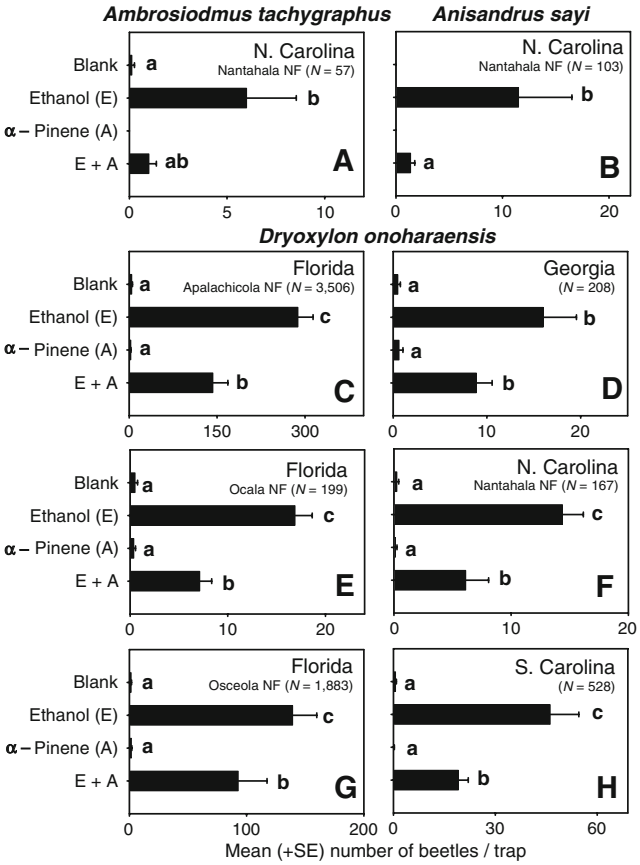

Fig. 3 Effects of ethanol and (−)-α-pinene on trap catches of *A. tachygraphus* (**a**), *A. sayi* (**b**), and *D. onoharaensum* (**c**–**h**; Scolytidae) in the southeastern US. Means followed by the same *letter* are not significantly different (α=0.05, Holm–Sidak multiple comparison) for three to four treatments (**a** and **c**–**h**) or by *t* test for two treatments (**b**). Treatments without a *letter* had zero catches

1987; Phillips et al. 1988). We specifically identify (−)-α-pinene as one of the active components for *D. terebrans*. Previously, Anderson (1977) found that α-pinene, technical pinene, and turpentine increased the attractiveness of loblolly pine logs to *I. grandicollis*. In Ontario, Chénier and Philogène (1989) captured more *I. grandicollis* in traps baited with lures that included α-pinene (total catch was only 25 beetles over six treatments). In Wisconsin, Erbilgin and Raffa (2000) found that *I. grandicollis* were attracted to traps baited with (−)-α-pinene. We know of no prior data on the specific attraction of *H. tenuis*, *H. porculus*, *H. salebrosus*, *X. ferrugineus*, and *X. pubescens* to α-pinene.

Attraction of bark beetles to monoterpenes is a fairly common phenomenon, especially in combination with pheromones (Seybold et al. 2006). Monoterpenes are common constituents of the air within forested stands, changing constantly due to various environmental and anthropogenic factors (Seybold et al. 2006). Compounds such as α-pinene are abundant in the resin of coniferous trees, particularly pine species (Smith 2000), providing important defenses against invasion by insects and diseases (Franceschi et al. 2005; Seybold et al. 2006). Leakage of monoterpenes into the environment around trees may occur through the outer bark and foliage of healthy trees, perhaps increasingly so from physically damaged trees or from trees weakened by factors such as drought or disease, which may indicate suitable breeding opportunities for bark and ambrosia beetles (Byers 1989; Franceschi et al. 2005; Raffa et al. 2005; Seybold et al. 2006). In addition, trees under attack by bark beetles release monoterpenes through the flow of resin and from the frass produced by boring beetles, thereby providing further attractiveness to other beetles (Seybold et al. 2006).

There are hundreds of volatile compounds in tree resins, and it is likely that bark beetles are attracted to monoterpenes other than α-pinene within this "dynamic aerial sea of monoterpenes" (Seybold et al. 2006). In felled loblolly pine, α-pinene is the dominant monoterpene in tree resin with four additional monoterpenes (myrcene, camphene, limonene, and β-pinene) present at levels of >5%

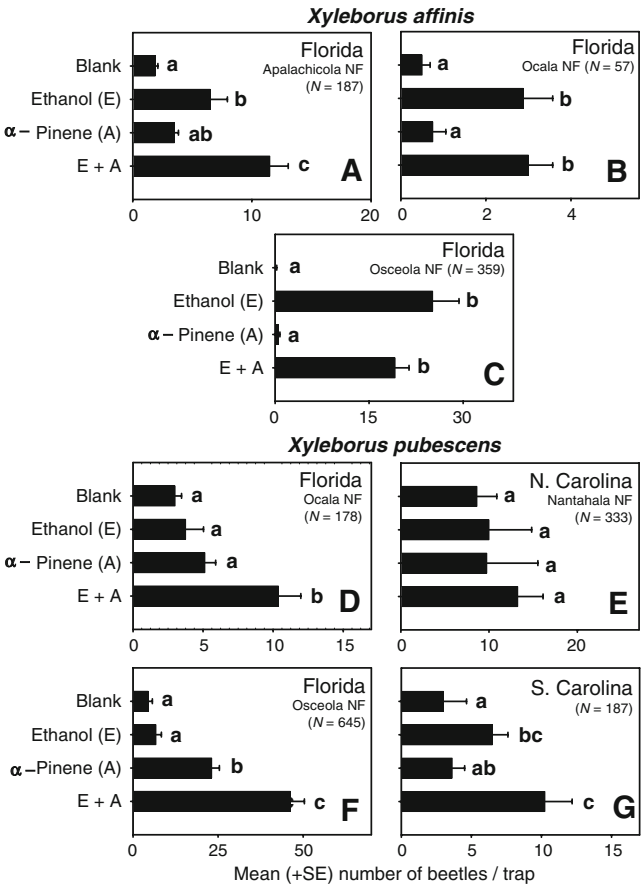

Fig. 4 Effects of ethanol and (−)-α-pinene on trap catches of *X. affinis* (**a**–**c**) and *X. pubescens* (**d**–**g**; Scolytidae) in the southeastern US. Means followed by the same *letter* are not significantly different (α=0.05, Holm–Sidak multiple comparison)

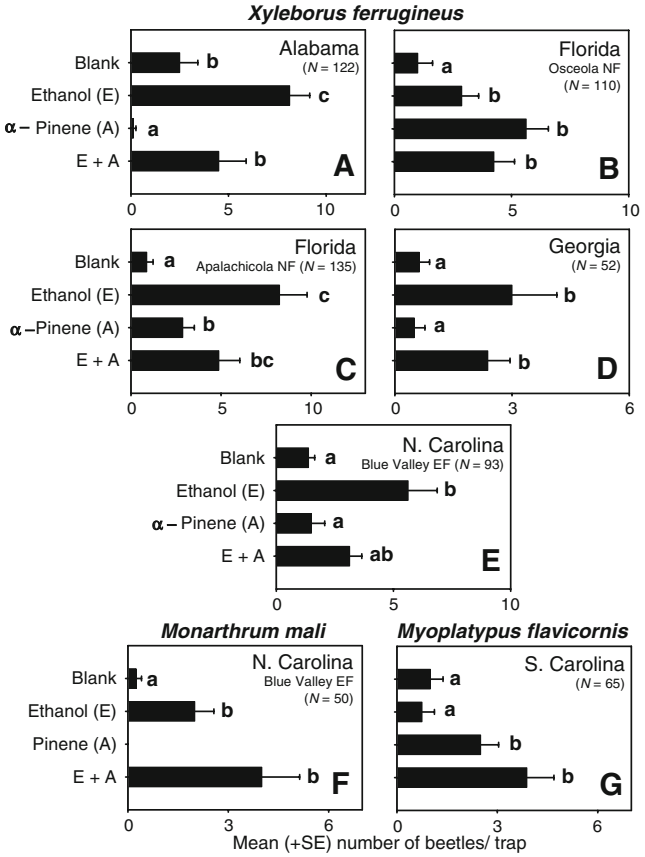

Fig. 5 Effects of ethanol and (−)-α-pinene on trap catches of *X. ferrugineus* (**a**–**e**), *M. mali* (**f**; Scolytidae), and *M. flavicornis* (**g**; Platypodidae) in the southeastern US. Means followed by the same *letter* are not significantly different (α=0.05, Holm–Sidak multiple comparison) for three to four treatments. Treatments without a *letter* had zero catches

(Werner 1972). In addition to α-pinene, *I. grandicollis* responded to myrcene, camphene, and limonene in walking bioassays (Werner 1972).

The issue of enantiomeric composition of α-pinene may be important as well (Seybold et al. 2006). We used (−)-α-pinene in our studies. Enantiomeric composition of α-pinene in pine trees can vary widely among pine species (Mirov 1961), although the relationship between enantiomeric composition and bark beetle responses may not be clear (Seybold et al. 2006). In California, Hobson et al. (1993) found that (+)-α-pinene was attractive to *D. valens* with attraction to (+)-α-pinene interrupted by (−)-α-pinene. (+)-α-Pinene is predominant in species such as *Pinus flexilis* James, *Pinus lambertiana* Dougl., and *Pinus resinosa* Ait., whereas (−)-α-pinene is the most common enantiomer in species such as *Pinus monticola* Dougl., *Pinus coulteri* D. Don, and *Pinus contorta* Dougl. ex Loud. (Mirov 1961), all of which occur within the range of *D. valens* and can be used as host material by *D. valens* (Furniss and Carolin 1977).

In another finding relevant to our first objective, traps baited with ethanol were attractive to the ambrosia beetles, *A. tachygraphus*, *A. sayi*, *D. onoharaensum*, *M. mali*, *X. saxesenii*, *X. affinis*, *X. ferrugineus*, *X. pubescens*, *X. crassiusculus*, *X. compactus*, and *X. germanus* (Figs. 1, 2, 3, 4, and 5), and the bark beetles, *C. heveae* and *Hypothenemus* sp. (Fig. 6a, b). Previously, Klimetzek et al. (1986) found that *X. saxesenii* was attracted to ethanol released at rates >1 mg/h whereas Phillips et al. (1988) found that *X. affinis*, but not *X. pubescens*, were attracted to traps baited solely with ethanol. Montgomery and Wargo (1983) state that ethanol was attractive to *M. mali* and *X. saxesenii* in oak forests in Connecticut (USA), although data were not presented. In southern California, *X. saxesenii* is attracted to ethanol-baited traps (Flint et al. 2007).

We know of no other prior data on the ethanol-based attraction of the ambrosia beetle species caught in our study. Yet, ethanol-baited traps are commonly (and successfully) used to assess temporal and spatial patterns of ambrosia beetles as well as in detection programs like CAPS and EDRR. For example, Roling and Kearby (1975) used ethanol-baited window traps to successfully monitor the seasonal flight patterns of *A. sayi*, *M. mali, X. saxesenii*, and *X. ferrugineus* in stands of oak in Missouri (US). Although no data are presented, they noted that previous attempts without ethanol were unsuccessful. Similarly, Markalas and Kalapanida (1997) used ethanol-baited traps to monitor flight periods of *X. saxesenii* in an oak forest in Greece. Ethanol-baited traps are used to monitor the flight activity of ambrosia beetles such *X. saxesenii*, *X. crassiusculus*, and *X. germanus* in horticultural tree nurseries prior to initiating an insecticide control program (Oliver and Mannion 2001; Bambara et al. 2008). Coyle et al. (2005) used ethanol-baited funnel traps to monitor flight activity of 28 species of bark and ambrosia beetles in stands of cottonwoods in South Carolina. Our data simply add evidential weight to the experiences of professionals in support of ethanol-baited traps for ambrosia beetles.

The attraction of ambrosia beetles to ethanol is likely related to their preference for woody material that has aged for a sufficient period of time for anaerobic respiration to generate ethanol within the tissues (Graham 1968; Cade et al. 1970; Moeck 1970; Lindelöw et al. 1993). Kelsey (1994) found that the ambrosia beetles *G. retusus* and *T. lineatum* preferred Douglas fir logs, *P. menziesii* (Mirb.) Franco, that had been felled in November of the previous year over logs that had been felled in January or March of the current year; ethanol concentrations were highest in logs felled in November of the previous year.

Hypoxia conditions arising in stressed trees also can lead to the production of ethanol and subsequent attack by bark and ambrosia beetles. Gara et al. (1993) found that lodgepole pines infected with heartwood decay fungi

Table 4 Significance levels for ANOVAs on effects of ethanol and (−)-α-pinene on bark beetle trap catches in the southeastern US

Species	Location	Rep	Ethanol (E)	α-Pinene (A)	ExA
Cryptocarenus heveae	FL—Ocala NF	0.810	<0.001	0.567	0.455
Dendroctonus terebrans	FL—Apalachicola NF	0.993	0.826	<0.001	0.826
	FL—Ocala NF	0.362	0.027	<0.001	0.216
	FL—Osceola NF	0.896	0.131	<0.001	0.956
	GA—Oconee NF	0.806	0.596	<0.001	0.831
	SC—Sumter NF	0.437	0.402	<0.001	0.085
Hylastes porculus	AL—Bankhead NF	0.006	0.008	<0.001	0.502
	GA—Oconee NF	0.091	<0.001	<0.001	0.008
	NC—Blue Valley EF	0.589	0.006	<0.001	0.002
	NC—Nantahala NF	0.147	0.189	<0.001	0.477
	SC—Sumter NF	0.678	0.440	<0.001	0.445
Hylastes salebrosus	AL—Bankhead NF	0.004	0.014	<0.001	0.463
	FL—Apalachicola NF	0.086	<0.001	<0.001	<0.001
	FL—Ocala NF	0.523	<0.001	<0.001	0.011
	FL—Osceola NF	0.165	<0.001	<0.001	<0.001
	GA—Oconee NF	0.819	0.006	<0.001	<0.015
	SC—Sumter NF	0.788	<0.001	<0.001	0.062
Hylastes tenuis	AL—Bankhead NF	0.700	0.870	<0.001	0.589
	FL—Apalachicola NF	0.467	0.738	<0.001	0.864
	GA—Oconee NF	0.981	0.913	<0.001	0.913
	NC—Blue Valley EF	0.163	0.446	<0.001	0.637
	NC—Nantahala NF	0.001	0.831	<0.001	0.573
	SC—Sumter NF	0.635	0.013	<0.001	0.301
Hypothenemus sp.	AL—Bankhead NF	0.003	<0.001	0.474	0.961
Ips grandicollis	AL—Bankhead NF	0.457	0.040	<0.001	0.040
	FL—Apalachicola NF	0.993	0.826	<0.001	0.826
	FL—Ocala NF	0.362	0.027	<0.001	0.216
	FL—Osceola NF	0.896	0.131	<0.001	0.956
	GA—Oconee NF	0.806	0.596	<0.001	0.831
	NC—Nantahala NF	0.024	0.775	<0.001	0.775
	SC—Sumter NF	0.437	0.402	<0.001	0.085
Pityophthorus cariniceps	NC—Blue Valley EF	0.888	0.001	<0.001	<0.001

emitted ethanol at rates higher than uninfected trees. Ethanol concentrations are higher in the roots and root collars of Douglas fir and ponderosa pine infected with root disease than in healthy uninfected trees (Kelsey and Joseph 1998; Kelsey et al. 1998). Ethanol production in Douglas fir branches increases with water stress, resulting in attacks by the Douglas fir engraver, *S. unispinosus* LeConte, in Oregon (Kelsey and Joseph 2001). Ethanol production and landing preferences by bark and wood-boring beetles in ponderosa pine are correlated strongly with stress from crown scorch caused by wildfires (Kelsey and Joseph 2003).

In answer to our second objective, we found that the binary combination of ethanol and (−)-α-pinene was preferred by *H. salebrosus* and *P. cariniceps* (Fig. 10). Previously, Phillips (1990) found that *H. salebrosus* preferred the combination of turpentine with ethanol to turpentine alone. However, the benefit of adding ethanol to (−)-α-pinene-baited traps was inconsistent for several other species among locations. The binary combination was preferred by *D. terebrans* at only one of six locations (Fig. 6e), *X. affinis* at one of three locations (Fig. 4a), *X. pubescens* at two of four locations (Fig. 4d, f), *I. grandicollis* at one of eight locations (Fig. 7a), *H. tenuis* at one of six locations (Fig. 8c), and *H. porculus* at two of five locations (Fig. 9c, e). Fatzinger et al. (1987) and Phillips et al. (1988) found that *D. terebrans* and *X. pubescens* in Florida preferred the binary combination of ethanol and turpentine, whereas *X. affinis* was unaffected by the addition of turpentine to ethanol-baited traps. In southern California, Flint et al. (2007) found that *H. tenuis* preferred traps baited with ethanol and (−)-α-pinene over those baited with either ethanol or (−)-α-pinene alone. We know of no other prior data on the specific attraction of these species to the combination of ethanol and (−)-α-pinene.

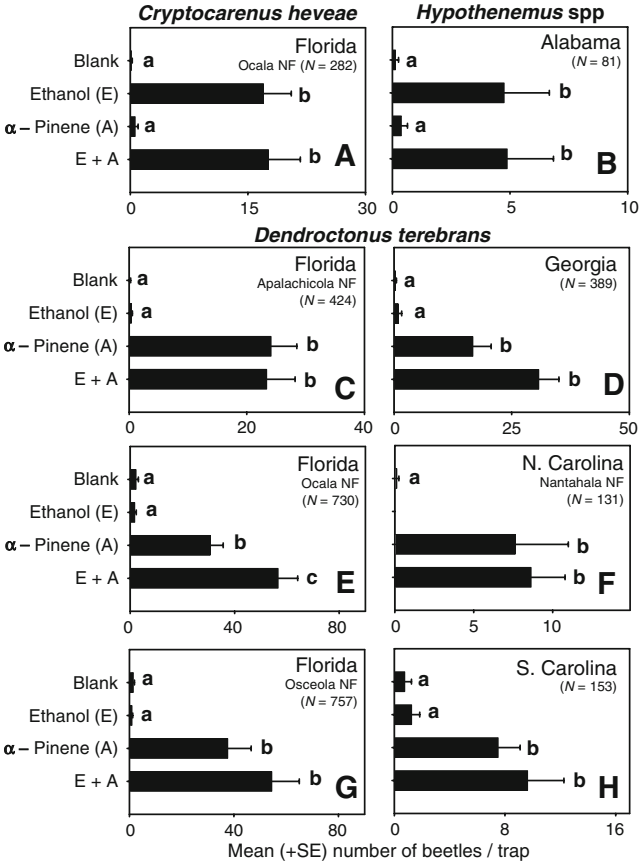

Fig. 6 Effects of ethanol and (−)-α-pinene on trap catches of *C. heveae* (**a**), *Hypothenemus* sp. (**b**), and *D. terebrans* (**c**–**h**; Scolytidae) in the southeastern US. Means followed by the same *letter* are not significantly different (α=0.05, Holm–Sidak multiple comparison)

In contrast, (–)-α-pinene had an interruptive effect on the responses of some species of ambrosia beetles to traps baited with ethanol. Catches of the ambrosia beetles *A. sayi* and *D. onoharaensum* to ethanol-baited traps were reduced by the addition of (−)-α-pinene (Fig. 3b–h). The exotic ambrosia beetle *D. onoharaensum* was one of the most common species in our trapping studies (Table 1). In two of eight locations, (−)-α-pinene had an interruptive effect on the attraction of *X. saxesenii* to ethanol (Fig. 1e–f). In British Columbia (Canada), α-pinene interrupted catches of *X. saxesenii* in ethanol-baited traps (L. Humble, unpublished data). However, Flint et al. (2007) found that (−)-α-pinene had no interruptive effect on catches of *X. saxesenii* to ethanol-baited traps in southern California, similar to our results with *X. saxesenii* in six of eight locations (Fig. 1a–d, g, h). With the exception of *I. grandicollis* at the Ocala NF (Florida; Fig. 7e), ethanol did not interrupt the response of bark beetles to traps baited with (−)-α-pinene. Catches of *I. grandicollis* at the seven other locations were not interrupted by ethanol (Fig. 7a–d, f–h).

Preferences for various combinations of ethanol and (−)-α-pinene may reflect conditions found in suitable hosts. In damaged or severed trees, the emissions of monoterpenes are likely high initially, decreasing over time with effects of desiccation and oxidation. In contrast, ethanol emissions are likely low initially, increasing over time as a consequence of anaerobic respiration (Kelsey 1994). Preferences of some species to ethanol with no apparent effect of (−)-α-pinene may relate to broad host ranges that include hardwood tree species; in fact, some ambrosia beetle species may be more common in hardwood species (Jordal et al. 2001).

We are unable to explain the variation among locations in responses of some species to baited traps. Although the same experimental design was employed at all locations, it is possible that the power was lower at some locations than others, particularly for species with low total catches. In some locations, sample size may have been insufficient to detect significant differences where such differences truly existed. It is possible that background or competing sources of ethanol and/or (−)-α-pinene may have differed significantly among locations. In some areas, emissions from baits

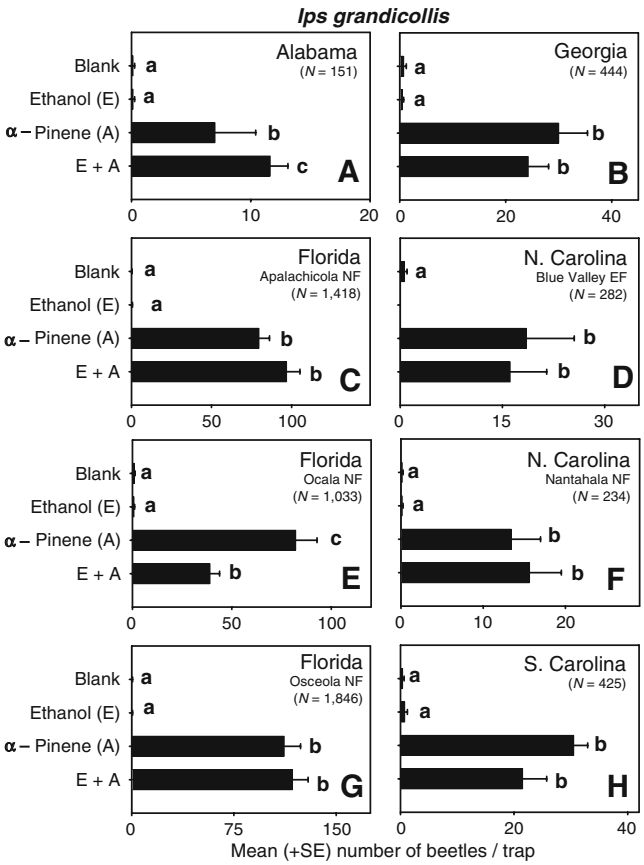

Fig. 7 **a**–**h** Effects of ethanol and (−)-α-pinene on trap catches of *I. grandicollis* (Scolytidae) in the southeastern US. Means followed by the same *letter* are not significantly different (α=0.05, Holm–Sidak multiple comparison)

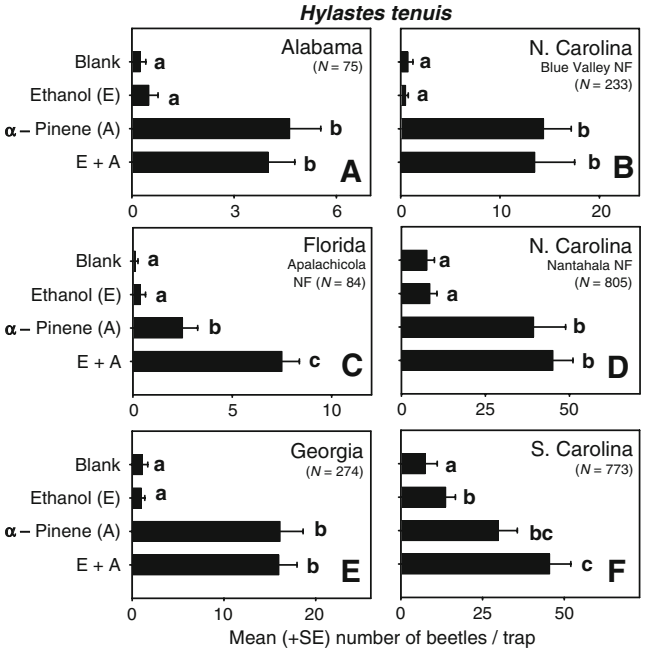

Fig. 8 **a**–**f** Effects of ethanol and (−)-α-pinene on trap catches of *H. tenuis* (Scolytidae) in the southeastern US. Means followed by the same *letter* are not significantly different (α=0.05, Holm–Sidak multiple comparison)

might have been overwhelmed by background levels of ethanol and/or (−)-α-pinene. In other areas, these emissions might have been dampened directly by vegetation. In addition to variation in trapping dates and primary pine

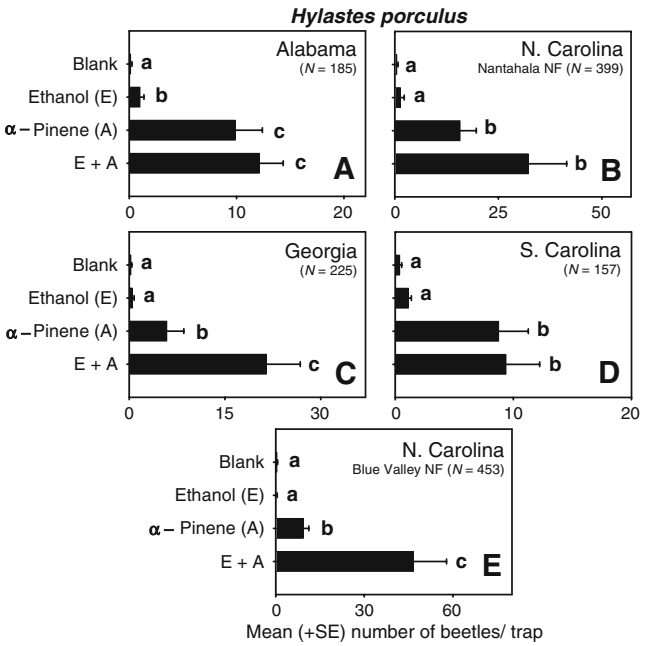

Fig. 9 **a**–**e** Effects of ethanol and (−)-α-pinene on trap catches of *H. porculus* (Scolytidae) in the southeastern US. Means followed by the same *letter* are not significantly different (α=0.05, Holm–Sidak multiple comparison)

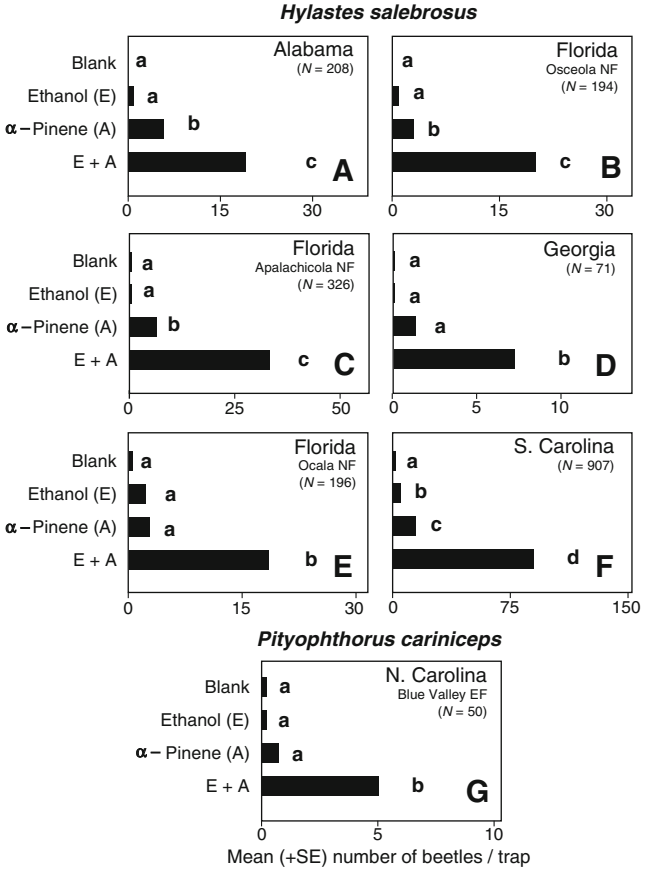

Fig. 10 Effects of ethanol and (−)-α-pinene on trap catches of *H. salebrosus* (**a**–**f**) and *P. cariniceps* (**g**; Scolytidae) in the southeastern US. Means followed by the same *letter* are not significantly different (α=0.05, Holm–Sidak multiple comparison)

species (Table 1), the eight study sites used in our study likely differed in many aspects including host composition, damage type, understory vegetation, and climate regimes. Unfortunately, we have no detailed information over an adequate sample size of locations to evaluate or explain this type of variation. Suffice it to say that our results suggest that variation in responses can be expected in an operational program as well.

In summary, we provide evidence to support the continued use of traps baited with ethanol and/or (−)-α-pinene in detection and monitoring programs for bark and wood-boring beetles. Moreover, the interruptive effect of (−)-α-pinene does necessitate the use of a separate trap baited solely with ethanol. Serendipitous to our stated objectives, we found a significant and disturbing pattern in the relative abundance of exotic species. Exotic non-native species of ambrosia beetles were consistently dominant in total trap catches of ambrosia beetles throughout our study area, accounting for 69.7–93.4% in North Carolina, 90.6% in Georgia, 82.9% in South Carolina, 90.0% in Alabama, and 75.1–96.6% in Florida.

The abundance of non-native ambrosia beetles in trap catches likely reflects their relative abundance in forested areas of the South. Previously in South Carolina and Tennessee, Coyle et al. (2005) and Oliver and Mannion (2001) found that the percentage of exotic beetles in ethanol-baited baited traps was 88% and 74%, respectively. Ambrosia beetles generally play an important role in initiating the decomposition cycle (Lindgren 1990). Yet, little is known of the impacts of exotic ambrosia beetles on forest ecology in southern forests, particularly with respect to decomposition of woody material and carbon sequestration. Moreover, the generalist host requirements and the incestuous nature of xyleborine ambrosia beetles suggest that new invasions and expansion of existing invasions are likely (Jordal et al. 2001; Hulcr et al. 2007), all without known consequences.

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Optimization of a Phenylacetaldehyde-Based Attractant for Common Green Lacewings (*Chrysoperla carnea s.l.*)

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Abstract In field trapping tests, the catch of *Chrysoperla carnea sensu lato* (Neuroptera: Chrysopidae) increased when acetic acid was added to lures with phenylacetaldehyde. The addition of methyl salicylate to the binary mixture of phenylacetaldehyde plus acetic acid increased catches even further. The ternary blend proved to be more attractive than β-caryophyllene, 2-phenylethanol, or 3-methyl eugenol (compounds previously described as attractants for chrysopids) on their own, and no influence on catches was recorded when these compounds were added as fourth components to the ternary blend. There were minimal changes in activity when (*E*)-cinnamaldehyde or methyl anthranylate (both evoking large responses from female or male antennae of *C. carnea* in this study) were added, although both compounds showed significant attraction on their own when compared to unbaited traps. In subtractive field bioassays with the ternary mixture, it appeared that the presence of either phenylacetaldehyde or methyl salicylate was important, whereas acetic acid was less so in the ternary mixture. The ternary blend attracted both female and male lacewings at sites in southern, central, and northern Europe. Possible applications of a synthetic attractant for lacewings are discussed.

Keywords Acetic acid · Attractant · *Chrysoperla* · Chrysopidae · Green lacewings · Methyl salicylate · Neuroptera · Phenylacetaldehyde

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Introduction

The common green lacewing, *Chrysoperla carnea sensu lato* (Neuroptera: Chrysopidae), is one of the most important lacewing species used in biological control. Adults of some genera and all lacewing larvae feed on pest aphids, scales, caterpillars, and other pests of many crops (McEwen et al. 2001). A synthetic attractant for green lacewings might be useful for monitoring lacewing abundance or manipulating lacewing population densities (for a review, see Szentkirályi 2002).

Lacewings of different genera (*Chrysopa* sp. and *Peyerimhoffina* sp.) are highly attracted to semiochemicals that lacewings produce themselves (Hooper et al. 2002). In *Chrysopa oculata* Say, a semiochemical apparently secreted by elliptical epidermal glands found only on the male abdominal sternites (Zhang et al. 2004) is a powerful aggregation pheromone (Chauhan et al. 2007). Although specific pheromones have yet to be found for lacewings in the genus *Chrysoperla*, several chemicals occurring in the

scent of several flowers attract *Chrysoperla* and other lacewings. 3-Methyl eugenol (Suda and Cunningham 1970; Umeya and Hirao 1975), methyl salicylate (Molleman et al. 1997; James 2003, 2006; James and Price 2004), β-caryophyllene (Flint et al. 1979), and 2-phenylethanol (Zhu et al. 1999, 2005) have all been reported to attract *C. carnea* or some other species of Chrysopidae in the field. Recently, we also discovered that phenylacetaldehyde was attractive to *C. carnea* in field tests in Hungary and Italy (Tóth et al. 2006). The objective of the present study was to optimize multicomponent attractant blends with phenylacetaldehyde as the core component. We investigated new, optimized attractant combinations and searched for synergists by using electroanntennographic and field screening techniques and by utilizing data from other synthetic lacewing attractants known from literature. In addition, the most attractive combination of volatiles was tested for its attractivity in several areas in Europe.

Methods and Materials

Electroantennograms Insects for electroantennogram (EAG) analyses were collected from the edge of an oak forest at Julianna major (Budapest, Hungary). To present the stimuli to the antenna during EAG studies, a stainless steel tube (Teflon-coated inside) with a constant humidified airflow of ca. 0.7 l/min was set up. An antenna was freshly amputated at the base from a live lacewing and mounted between two glass capillaries containing 0.1 N KCl solution. The mounted antenna was placed at ca. 3 mm distance from the out-coming airflow. One of the electrodes was grounded, while the other was connected to a high impedance DC amplifier (IDAC-232, Syntech, Hilversum, The Netherlands). Test compounds (10 μg each) were administered in hexane solution onto a 10×10 mm piece of filter paper inside a Pasteur pipette. Stimuli consisted of pushing 1 ml of air through the Pasteur pipette into the airstream flowing towards the antenna. Response amplitudes were normalized against the means of responses to phenethyl alcohol (eliciting medium high responses from antennae), which was tested before and after the test compounds. Stimuli were administered at ca. 20–30 s intervals.

Trap Types For field studies conducted in Hungary, we used standard CSALOMON® VARL+ funnel traps (www.julia-nki.hu/traps) produced by the Plant Protection Institute, HAS (Budapest, Hungary). This trap type was developed originally for capturing moths (Tóth et al. 2000; Subchev et al. 2003) but later proved to be suitable for the green lacewing (Tóth et al. 2006). The trap consists of an opaque plastic funnel (top opening outer diameter, 13 cm; funnel hole diameter, 3 cm; height of funnel, 16 cm), with a 20×20 cm flat plastic roof. The trap also has a round transparent plastic collection container (ca. 1 l capacity) attached to the bottom funnel by a rubber band. The bait was suspended from the middle of the roof and positioned slightly above the level of the upper edge of the large funnel opening. A small piece (1×1 cm) of household anti-moth strip (Chemotox®, Sara Lee, Temana Intl. Ltd, Slough, UK; active ingredient 15% dichlorvos) was placed in the collection container to kill the captured insects.

For trials conducted in Italy, we used sticky delta traps (the standard CSALOMON® RAG traps produced by Plant Protection Institute, HAS, Budapest, Hungary; Szöcs 1993; Tóth and Szöcs 1993; www.julia-nki.hu/traps). In Norway, we used delta traps with sticky bottoms produced by PheroNet AB, Lund, Sweden.

Baits All synthetic compounds (>95% chemical purity as per the manufacturer) were obtained from Sigma-Aldrich Kft. (Budapest, Hungary). For preparing the baits, compounds were loaded onto a 1-cm piece of dental roll (Celluron®, Paul Hartmann AG, Heidenheim, Germany), which was put into a polyethylene bag (ca. 1.0×1.5 cm) made of 0.02 mm linear polyethylene foil (FS471-072, Phoenixplast BT, Pécs, Hungary). Loaded dose of single compounds was kept at 100 mg each, unless otherwise stated in the respective experiments. The dispensers were heat sealed and attached to 8×1 cm plastic handles for easy handling when assembling the traps. Dispensers were wrapped singly in pieces of aluminum foil and stored at −18°C until used. In the field, baits were changed at 2 to 3-week intervals, as previous experience showed that they do not lose their attractiveness during this period (Tóth et al. 2006)

Field Tests Field comparison tests were conducted routinely by using the methods applied in similar studies on pheromones and attractants (e.g., Roelofs and Cardé 1977; Arn et al. 1986). Trapping experiments were conducted in fruit orchards. Green lacewings are frequently seen flying at 1.5–2.0 m in the crown of trees, thus traps were suspended in the trees at a height of 1.5–1.7 m above ground. One trap of each treatment was incorporated into a block so that individual treatments were 5–8 m apart (according to the distance between trees at the respective sites). Blocks were situated 15–20 m apart. Unless otherwise stated, five blocks of traps were applied in an experiment, and traps were inspected twice weekly. In Hungary, field tests were conducted at Halásztelek, in a sour cherry orchard, and at Érd-Elviramajor, in a cherry orchard (both sites situated in Pest county). In Italy, field tests were conducted in an apricot orchard near Rotondella (southern Italy), and in Norway, traps were placed in sweet cherry orchards in two

different areas in Ås (southeastern Norway) and Ullensvang (western Norway).

Experimental Details

Experiments 1A and 1B The objective of these preliminary tests was to study the influence of acetic acid added to phenylacetaldehyde. These tests were originally aimed at catching noctuid moths (Lepidoptera: Noctuidae). Treatments included traps baited with phenylacetaldehyde on its own [a well-known moth attractant (e.g., Creighton et al. 1973; Cantelo and Jacobson 1979)], or phenylacetaldehyde plus acetic acid (1:1). In the treatment containing both compounds in experiment 1A, acetic acid and phenylacetaldehyde were loaded into separate dispensers (so in this case, traps with this treatment contained two dispensers), whereas in experiment 1B, the two compounds were loaded into a single dispenser. Both tests were conducted at Halásztelek; experiment 1A was carried out from 9 May to 10 June, 2003, whereas experiment 1B was carried out from 10 June to 23 September, 2003. There were five blocks in the former and six blocks in the latter.

Experiment 2 The objective was to study the optimal ratio of acetic acid added to phenylacetaldehyde. Treatments included traps baited with phenylacetaldehyde or acetic acid alone, and their binary mixtures in 10:1, 1:1, and 1:10 ratios. The test was conducted at Halásztelek from 11 August to 24 August, 2003.

Experiment 3 In this test, we studied the activity of methyl salicylate or β-caryophyllene alone or in addition with the optimal blend of phenylacetaldehyde and acetic acid. Treatments included methyl salicylate or β-caryophyllene alone, the binary mixture of phenylacetaldehyde and acetic acid (1:1), ternary mixtures of phenyalcetaldehyde, acetic acid, and β-caryophyllene or methyl salicylate, and unbaited traps. The test was conducted at Érd-Elviramajor from 14 June to 23 July, 2004.

Experiment 4 This test was designed to study the influence of varying doses of methyl salicylate added to the optimal phenylacetaldehyde + acetic acid blend. Traps baited with methyl salicylate on its own, the binary phenylacetaldehyde + acetic acid blend, and ternary mixtures of phenylacetaldehyde + acetic acid + methyl salicylate (1:1:1, 10:10:1, 1:1:10) and unbaited controls were set out. An extra treatment which contained the phenylacetaldehyde + acetic acid blend plus methylsalicylate in separate dispensers was also tested. The test was conducted at Érd-Elviramajor from 23 July to 13 October, 2004.

Experiment 5 This test was aimed at comparing efficacy of the phenylacetaldehyde + acetic acid + methyl salicylate ternary blend with that of the known lacewing attractants 2-phenylethanol and 3-methyl eugenol. The test consisted of the treatments 2-phenylethanol and 3-methyl eugenol on their own, the ternary phenylacetaldehyde + acetic acid + methyl salicylate blend, and unbaited traps. The test was conducted at Érd-Elviramajor from 4 to 19 July, 2005.

Experiment 6 The objective was to study the influence of 2-phenylethanol and 3-methyl eugenol when added to the ternary phenylacetaldehyde + acetic acid + methyl salicylate blend. Treatments included the ternary phenylacetaldehyde + acetic acid + methyl salicylate mixture (1:1:1), quaternary blends of these compounds with 2-phenylethanol or 3-methyl eugenol added (1:1:1:1), a blend of all five compounds together (1:1:1:1:1), and unbaited traps. The test was conducted at Érd-Elviramajor from 19 July to 16 August, 2005.

Experiment 7 In this test, we studied the activity of cinnamaldehyde and methyl anthranylate, both compounds with high EAG activity. Traps with (*E*)-cinnamaldehyde or methyl anthranylate on their own were compared to traps with the ternary phenylacetaldehyde + acetic acid + methyl salicylate mixture, the quaternary blends containing (*E*)-cinnamaldehyde or methyl anthranylate as fourth components, and unbaited traps. The test was conducted at Halásztelek from 1 June to 13 July, 2007.

Experiments 8 and 9 The objective of these tests was to compare the relative importance of the single components in the ternary blend of phenylacetaldehyde, acetic acid, and methyl salicylate. In both experiments, treatments included phenylacetaldehyde on its own, its binary combinations with acetic acid or methyl salicylate (1:1), and the ternary combination (1:1:1). Experiment 9 also included unbaited traps. The tests were conducted at Érd-Elviramajor from 16 August to 12 September, 2005 (experiment 8) and at Halásztelek from 13 July to 21 August, 2007 (experiment 9).

Activity of the Optimized Attractant in Different European Regions Apart from the comparison tests performed in Hungary (central Europe), the optimized ternary attractant of phenylacetaldehyde + acetic acid + methyl salicylate (1:1:1) also was tested at sites in southern Europe (Italy) and in northern Europe (Norway). The purpose of these tests was to confirm attraction of *C. carnea* in these regions.

It Italy, five traps with bait dispensers and five unbaited control traps were checked for adult *C. carnea* weekly between 14th April and 27th July.

In Ullensvang (western Norway), ten traps with bait dispensers and ten unbaited control traps were checked for

adult *C. carnea* four times between 16th May and 15th June. From 16th June until 12th September 3, traps with bait dispensers and five control traps were checked for adults 12 times. Further, these traps were checked for *C. carnea* eggs twice between 16th May and 15th June (ten baited traps and ten control traps) and 12 times between 16th June and 12th September (five baited traps and five control traps). In Søråsjordet, Ås (southeastern Norway), five traps with bait dispensers and one unbaited control trap were checked ten times for both adults and eggs between 29th May and 8th August. A second field in this area, Norderåshagen, had seven traps and one control trap and was checked five times from 29th May until 13th June.

Identification of Species Within the C. carnea Group The widespread and abundant *C. carnea s.l.* is composed of several sibling, cryptic, and sympatric species (Henry et al. 2001). For their identification, we used several morphological traits of adults (Thierry et al. 1992, 1998; Henry et al. 1996, 2002, 2003). As we were aware of the debates about a valid nomenclature within the *C. carnea* group (Canard and Thierry 2005), we accepted the species names given by Henry et al. (1996, 2002, 2003).

Statistical Analyses The data units for the field experiments were number of insects caught/inspection. The catches from field trapping tests were transformed by using $(x+0.5)^{1/2}$ as suggested by Roelofs and Cardé (1977) for similar experiments. In many field trapping tests, trap catches are below ten or even zero, so this or a similar transformation is desirable (Tukey 1949, 1955). Data were analyzed by a Student's *t* test (two treatments only) or analysis of variance (ANOVA; more than two treatments). Treatment means were separated by the Games–Howell Test (Games and Howell 1976; Jaccard et al. 1984). Where one of the treatments caught no insects, the Bonferroni–Dunn test (Dunn 1961) was used to check that mean catches in other treatments were not significantly different from zero catch (see also table and figure legends). To analyze EAG responses, we used ANOVA followed by Fisher's protected least significant difference (LSD) for significance levels (Fisher 1966). All statistical procedures were conducted by using the software packages StatView® v4.01 and SuperANOVA® v1.11 (Abacus Concepts, Berkeley, CA, USA).

Results

EAG Response Spectra Both female and male antennae showed similar response intensities to the range of compounds tested (Fig. 1). The largest responses were evoked by (*E*)-cinnamaldehyde, and these responses were significantly different from all other compounds. This was followed in tendency by responses to methyl anthranylate, methyl salicylate, β-ionone, benzyl alcohol, and 2-phenylethanol, but responses to these compounds were not significantly higher than those to the phenethyl alcohol standard. Phenylacetaldehyde evoked medium large responses similar to those of the standard. The rest of the compounds evoked rather uniform, low to medium EAG responses.

Field Comparison Tests, Hungary In experiment 1A, traps baited with the binary combination of phenylacetaldehyde and acetic acid caught significantly more green lacewings than traps with only phenylacetaldehyde throughout the test period. Mean catches/trap/inspection (±SE) were 3.31±0.60 vs. 1.56±0.37, respectively (P=0.011 by Student's *t* test). Similar results were recorded in experiment 1B, the respective mean catches were 6.69±0.41 vs. 4.70±0.34 specimens (P=0.002 by Student's *t* test). Unbaited control traps caught a mean (±SE) of 0.00±0.00 (experiment 1A) or 0.02±0.01 (experiment 1B) lacewings, respectively.

In the ratio test (experiment 2), traps with the 1:1 blend of phenylacetaldehyde + acetic acid caught the highest number of *C. carnea*, and mean catch in these traps was significantly higher compared to traps with phenylacetaldehyde alone (Fig. 2). The 10:1 blend produced intermediate catches. Very little was captured with the 1:10 blend, and zero catches were recorded in traps with acetic acid only.

The known lacewing attractant β-caryophyllene (Flint et al. 1979) did not catch more than unbaited controls and did not influence catches when added to the binary phenylacetaldehyde + acetic acid combination (Table 1, experiment 3). Methyl salicylate (Molleman et al. 1997; James 2003) was also inactive on its own; however, it increased catches significantly when added to the binary phenylacetaldehyde + acetic acid blend (Table 1, experiment 3).

The synergistic action of methyl salicylate was confirmed in experiment 4 (Fig. 3). Addition of methyl salicylate in equal or ten times lower amounts compared to the other two compounds in the ternary blend produced higher catches than the binary phenylacetaldehyde + acetic acid mixture. Similarly high catches were observed when methyl salicylate was added in a separate dispenser or loaded together in the same dispenser. When the amount of phenylacetaldehyde + acetic acid was decreased in the ternary blend, catches decreased. Methyl salicylate on its own again showed no activity (Fig. 3).

When comparing efficacy of the 1:1:1 ternary blend with that of the described lacewing attractants 2-phenylethanol (Zhu et al. 1999, 2005) and 3-methyl eugenol (Suda and Cunningham 1970; Umeya and Hirao 1975), the ternary blend caught approximately one magnitude more than the single compounds, although both 2-phenylethanol and 3-

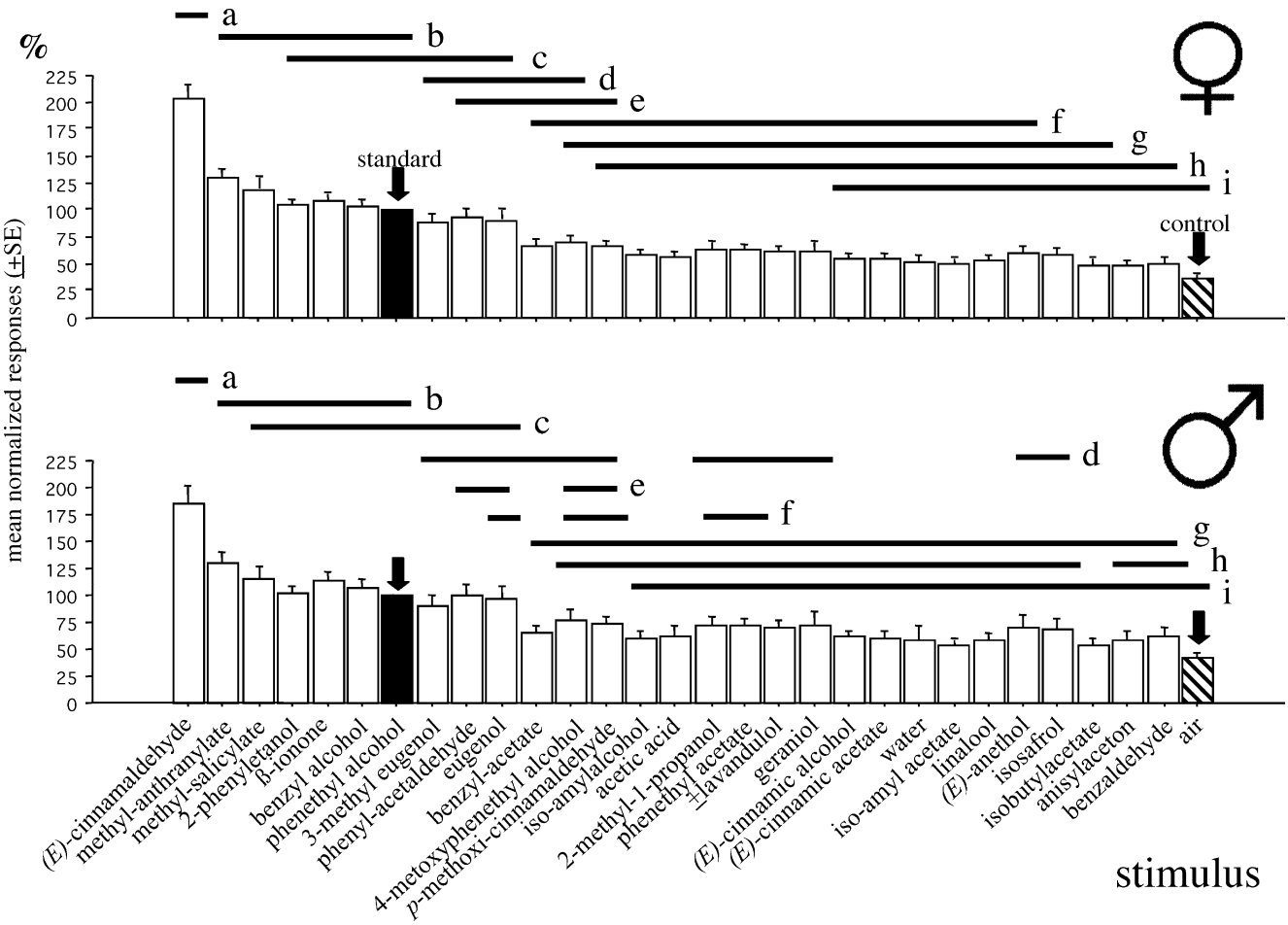

Fig. 1 Normalized EAG responses of female or male green lacewing antennae to a range of attractant candidate compounds. *Columns* show mean responses of antennae from nine female or 11 male insects tested. Responses were normalized to the response evoked by the standard phenethyl alcohol. *Columns with horizontal bars* not significantly different (α=0.05) by ANOVA, Fisher's Protected LSD test

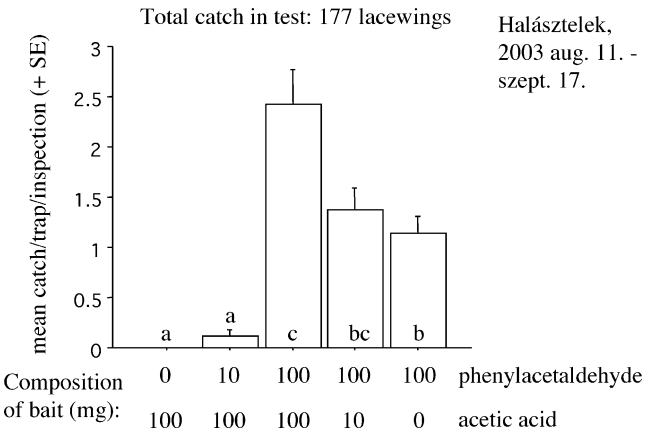

Fig. 2 Mean catches of green lacewings in traps baited with blends in different ratios of phenylacetaldehyde and acetic acid or with the single compounds (experiment 2; total caught in test, 177 lacewings). *Columns with same letter* not significantly different (α=0.05) by ANOVA, Games–Howell test. Significant difference from zero catch (acetic acid on its own) was tested by Bonferroni–Dunn test

methyl eugenol showed significantly increased catches as compared to unbaited controls (Table 1, experiment 5). However, when these compounds were added to the ternary blend, no influence on catches was observed (Table 1, experiment 6).

Finally, when the activity of (*E*)-cinnamaldehyde and methyl anthranylate, both evoking high EAG responses from antennae of both sexes (Fig. 1) were studied, the single compounds showed significant attraction as compared to the unbaited traps (Table 1, experiment 7). However, their addition to the ternary blend did not increase catches. In fact, the quaternary mixture containing methyl anthranylate caught less than the ternary blend.

Subtraction tests aimed to study the relative importance of the components of the ternary phenylacetaldehyde + acetic acid + methyl salicylate blend yielded similar trends of catches in both experiment 8 and 9 (Fig. 4). Lowest catches were recorded with the acetic acid + methyl salicylate binary combination; however, catches in these traps were higher than in unbaited traps (in experiment 9, where unbaited traps

Table 1 Catches of green lacewings, *Chrysoperla* spp., in traps baited with synthetic attractant candidates and their mixtures or in unbaited traps at various sites in Europe

Bait composition (mg)								Mean catch/trap/inspection			
Phenylacet-aldehyde	Acetic acid	Methyl salicylate	β-Caryo-phyllene	2-Phenyl-ethanol	3-Methyl eugenol	Cinnam-aldehyde	Methyl anthranylate	Experiment 3	Experiment 5	Experiment 6	Experiment 7
100	100	–	–	–	–	–		4.90b	n.t.	n.t.	n.t.
100	100	100	–	–	–	–	–	7.21c	8.95a	4.38a	10.02d
–	–	100	–	–	–	–	–	0.32a	n.t.	n.t.	n.t.
100	100		100		–	–	–	3.39b	n.t.	n.t.	n.t.
–	–	–	100		–	–	–	0.03a	n.t.	n.t.	n.t.
–	–	–	–	100	–	–	–	n.t.	1.15b	n.t.	n.t.
–	–	–	–		100	–	–	n.t.	1.05b	n.t.	n.t.
100	100	100	–	100	–	–	–	n.t.	n.t.	5.43a	n.t.
100	100	100	–		100	–	–	n.t.	n.t.	4.91a	n.t.
100	100	100	–	100	100	–	–	n.t.	n.t.	4.66a	n.t.
–	–	–	–	–	–	100	–	n.t.	n.t.	n.t.	4.78c
100	100	100	–	–	–	100	–	n.t.	n.t.	n.t.	11.08d
–	–	–	–	–	–	–	100	n.t.	n.t.	n.t.	2.22b
100	100	100	–	–	–	–	100	n.t.	n.t.	n.t.	2.93b
–	–	–	–	–	–	–	–	0.09a	0.10c	0.03b	0.06a
Total number of lacewings caught in test:								1822	225	675	1864

Means with same letter within one column are not significantly different (α=0.05) by ANOVA, Games–Howell

n.t. not tested

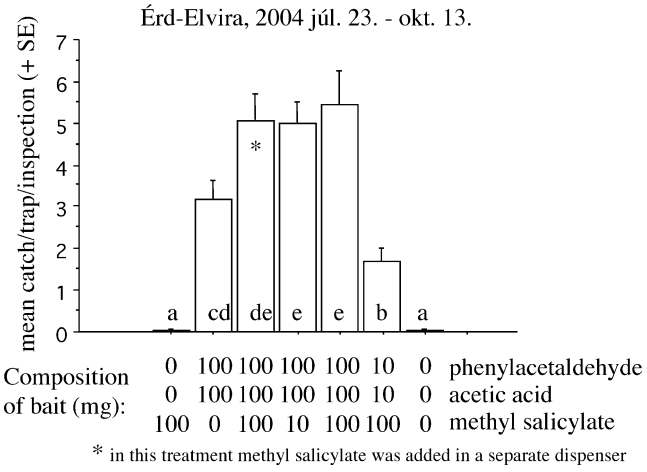

Fig. 3 Mean catches of green lacewings in traps baited with blends of phenylacetaldehyde, acetic acid, and methyl salicylate in different ratios or with the single compounds (experiment 4; total caught in test, 1,210 lacewings). *Columns with same letter* not significantly different (α=0.05) by ANOVA, Games–Howell test

were also operated). Phenylacetaldehyde on its own produced medium high catches. The binary phenylacetaldehyde + acetic acid blend showed a tendency of catching more than phenylacetaldehyde on its own. Largest catches were observed in the binary phenylacetaldehyde + methyl salicylate mixture or the ternary blend, and no significant difference was found between these blends.

Among randomly selected specimens caught in experiments 8 and 9 (138 and 941, respectively), 30.0% and 62.2% were females. There was no apparent difference in ratio of sexes caught among the different treatments.

A determination effort of a random sample of 205 (experiment 8, 92% out of a total of 222 captured) and 559 (experiment 9, 50.4% out of a total of 1,109 captured) lacewing specimens resulted in 84.1% and 49.1% of *C. carnea* s. str. (Stephens), 11.6% and 39.1% of *Chrysoperla lucasina* Lacroix, 4.3% and 9.9% of *Chrysoperla pallida* (Henry et al. 2002), respectively. The remaining 3.5% of specimens from experiment 9 were morphologically similar to *Chrysoperla agilis* (Henry et al. 2002); however, the occurrence of this species in the Hungarian fauna has not been reported previously. To sum it all up, all captured specimens investigated belonged to the *C. carnea* group.

Activity of the Optimized Attractant in Different European Regions In Rotondella, a total of 128 adult *C. carnea* were caught (83 females, 16 males, and 29 unsexed specimens) in traps with the ternary lure between 14th April and 27th July (16 inspections on five traps) resulting in a mean (±SE) of 1.60±0.18 adults/trap/inspection. Only five adults (four females and one male) were found in unbaited control traps.

In Ullensvang, Western Norway, a total of 18 adult *C. carnea* were caught in traps with the ternary lure between 16th May and 15th June (four inspections on ten traps) resulting in a mean (±SE) of 0.45±0.19 adults/trap/inspection. Between 16th June and 12th September, a total of 14 adults were caught (12 inspections on three traps) resulting in a mean (±SE) of 0.23±0.10. Further, a total of 287 eggs were found inside ten delta traps with baits between 16th May and 15th June, and a total of 26 eggs were found inside five delta traps with baits between 16th June and 12th September. No adults or eggs were found in

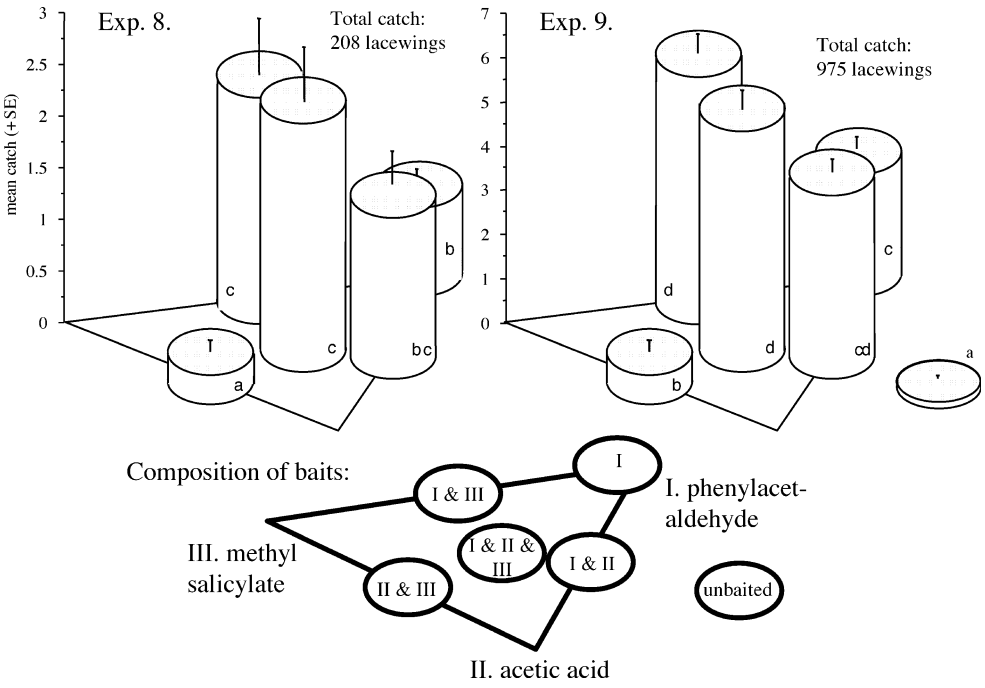

Fig. 4 Mean catches of green lacewings in traps baited with binary and ternary blends of phenylacetaldehyde, acetic acid, and methyl salicylate or with phenylacetaldehyde by itself (experiments 8 and 9; total caught in tests, 208 and 975 lacewings, respectively). *Columns with same letter* not significantly different (α=0.05) by ANOVA, Games–Howell test

unbaited control traps. In Søråsjordet, Ås (southeastern Norway), a total of 63 adults were caught in delta traps with the ternary lures between 29th May and 8th October (10 inspections on 5 traps) resulting in a mean (±SE) of 1.26± 0.22 adults/trap/inspection. In Norderåshagen, Ås (five inspections on seven traps), a total of 137 adults were caught between 29th May and 13th June (3.91±0.45 adults/trap/inspection). A total number of 99 and 156 eggs were found in the two fields, respectively, inside the traps during the corresponding periods. In the unbaited control traps, a total of one adult and two eggs were found.

Discussion

In this study, the addition of acetic acid clearly enhanced the behavioral activity of the previously known synthetic attractant phenylacetaldehyde. Acetic acid is known to be weakly attractive to several insect species (i.e., Yothers 1927; Keiser et al. 1976; Casana-Giner et al. 1999). Attractant combinations of acetic acid and other food- or host-derived volatile compounds have been reported to show increased activity in yellowjacket wasps (Landolt 1998), in noctuid or pyralid moths (Landolt and Alfaro 2001; Tóth et al. 2002; Landolt 2005), and, more recently, in the codling moth (Landolt et al. 2007). The presence of acetic acid usually is thought to indicate fermentation products, and attraction to acetic acid-emitting sources has been explained as orientation towards food sources by insects that feed on fermenting materials (Utrio and Eriksson 1977; Landolt et al. 2007). A similar hypothesis can explain the increased response of green lacewings to blends containing acetic acid.

Phenylacetaldehyde will produce (*E*)-2,4-diphenylbut-2-enal in the presence of acetic acid (Goetz et al. 1990). This reaction can be expected to take place when both compounds are loaded into the same dispenser. Because the addition of acetic acid resulted in a significant increase in catches both when the two compounds were presented in two different dispensers (experiment 1A) or when they were loaded into the same dispenser (experiment 1B), it suggests that the possible formation of (*E*)-2,4-diphenylbut-2-enal had no dramatic effect on behavioral activity. In this study, no attempt was made to examine in detail the possible influence of (*E*)-2,4-diphenylbut-2-enal on the activity of the phenylcetaldehyde + acetic acid blend, but this interaction should be clarified in future experiments.

We demonstrated in this work that trap catches to the binary mixture of phenylacetaldehyde and acetic acid can be further increased by the addition of methyl salicylate. This compound attracted *C. carnea* green lacewings in a test originally aimed at predators of the pear psylla (Molleman et al. 1997) and was also attractive to *Chrysopa nigricornis* Burmeister (James 2003). We did not observe any significant attraction to methyl salicylate on its own (Table 1, Fig. 3).

The compound β-caryophyllene has earlier been described as an attractant for *C. carnea* (Flint et al. 1979), but we did not confirm these findings. Caryophyllene has also been found to be behaviorally inactive in other studies (Zhu et al. 2005; Tóth et al. 2006). The reason for this discrepancy in attraction is not clear.

3-Methyl eugenol, which has been reported as attractive for some chrysopid spp. (Suda and Cunningham 1970; Umeya and Hirao 1975), showed some activity with *C. carnea* when presented alone in our tests. The attraction of *C. carnea* to 2-phenylethanol presented alone (e.g., Zhu et al. 2005) was also confirmed in our study. However, none of the above described compounds increased catches of *C. carnea* when added to the ternary blend optimized in this study.

We screened synthetic compounds by EAG to look for attractant candidates, as suggested by Dodds and McEwen (1998). Both compounds that evoked the highest responses, (*E*)-cinnamaldehyde and methyl anthranylate, were attractive in the field when presented alone. To our knowledge, neither has been reported to be attractive to lacewings. However, when (*E*)-cinnamaldehyde was added to the ternary blend optimized in this study, we observed no impact on the trap catches. Addition of methyl anthranylate possibly reduced catches (although this phenomenon should be confirmed through further testing).

There are other potential semiochemicals that might bear further study. For example, in a laboratory olfactometer bioassay, Van Emden and Hagen (1976) reported that tethered female green lacewings spent ca. 70% of the total time flying towards reaction mixtures thought to produce indole acetaldehyde, which led the authors to suggest that indole acetaldehyde was a lacewing attractant. However, Van Emden and Hagen (1976) never tested the synthesized pure compound in the laboratory or in the field.

Based on our results, it appears that the ternary blend described in this study is the most powerful synthetic attractant reported to date for *C. carnea*. Green lacewing populations from southern to northern and central Europe responded equally well in this study, and there was no indication of geographical differences in response, so this ternary attractant may prove to have widespread applicability. It remains to be seen whether *C. carnea* populations outside of Europe will also be as responsive.

From the subtraction tests of the ternary blend (experiments 8 and 9), it appears that the removal of phenylacetaldehyde had the most impact on the activity, whereas the removal of methyl salicylate resulted in a quantitative but not significantly lower reduction in trap catch. Removal of acetic acid did not lower trap catch. The combination of

methyl salicylate and phenylacetaldehyde was two to three times more attractive than phenylacetaldehyde alone was. More detailed studies should be carried out to evaluate whether acetic acid can be omitted altogether without seriously affecting efficiency.

Most insect predators show omnivorous feeding behavior, i.e., by feeding directly or indirectly on plant materials such as pollen or nectar (Jervis and Kidd 1996; Villenave et al. 2005). Green lacewings are adapted to unpredictable food supplies and larval habitats. Adult females perform obligatory migration flights where they do not react to olfactory stimuli. After the initial migration flight, females start responding to plant odors by a combination of appetitive down- and upwind flights (Duelli 1980, 2001).

The three "cryptic" lacewing species, *C. lucasina*, *C. pallida*, and *C. carnea* s. str. captured in baited traps in this study all belong to the *C. carnea* species complex. No other chrysopids were caught in significant numbers. An explanation could be that floral food sources are more important for green lacewing adults of genera *Chrysoperla* (and probably also for *Dichochrysa*), whose diet regime is palynoglycophagous, than for other chrysopids (Canard 2001).

The attractant described in this study appears to be highly attractive to females, which is advantageous from the point of view of applicability in biological control efforts. *C. carnea* females attracted by our ternary attractant may spend a long time in the vicinity of the bait dispenser, searching for a food source and oviposition sites. Preliminary observations of eggs deposited in traps indicate that the ternary attractant may give rise to major bouts of oviposition. If confirmed in more extensive experiments, this response may be advantageous in biological control. A large number of lacewing larvae will reduce aphid populations on that particular plant. The ternary attractant might also increase biological control by increasing lacewing numbers in overwintering boxes, a method widely used by organic growers.

In the closely related *C. oculata*, the synthetic main component of the aggregation pheromone, (1*R*,2*S*,5*R*,8*R*)-iridodial, attracted many males into traps (Zhang et al. 2004). More recently, Chauhan et al. (2007) reported that although few females entered traps baited with iridodial, they found many female insects in the vicinity of the traps. As the pheromonal communication and host attraction systems of the *C. carnea* complex become better known, combinations of pheromones with floral attractants may provide optimal management tools to exert better biological control by this group of beneficial insects.

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The Role of Extrafloral Nectar Amino Acids for the Preferences of Facultative and Obligate Ant Mutualists

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Abstract Plants in some 300 genera produce extrafloral nectar (EFN) to attract ants as a means of indirect defence. Among Mesoamerican *Acacia* species, obligate myrmecophytes produce EFN constitutively to nourish symbiotic ant mutualists, while non-myrmecophytes induce EFN secretion in response to herbivore damage to attract non-symbiotic ants. Since symbiotic *Acacia* ants entirely depend on the host-derived food rewards while non-symbiotic ants need to be attracted to EFN, this system allows comparative analyses of the function of EFN components in ant nutrition and attraction. We investigated sugar and amino acid (AA) composition in EFN of two myrmecophytes (*Acacia cornigera* and *Acacia hindsii*) and two related non-myrmecophyte species (*Acacia farnesiana* and *Prosopis juliflora*). AA composition allowed a grouping of myrmecophytes vs. non-myrmecophytes. Behavioural assays with obligate *Acacia* inhabitants (*Pseudomyrmex ferrugineus*) and non-symbiotic ants showed that AA composition affected ant preferences at high but not at low AA/sugar ratios. Most interestingly, behavioural responses differed between the two types of ants tested: Symbiotic ants showed a clear preference for higher AA concentrations and preferred nectar mimics with those four AAs that most significantly characterised the specific nectar of their *Acacia* host plant. In contrast, non-symbiotic ants distinguished among nectars containing different sugars and between solutions with and without AAs but neither among nectars with different AA/sugar ratios nor among mimics containing different numbers of AAs. Our results confirm that both AAs and sugars contribute to the taste and attractiveness of nectars and demonstrate that the responses of ants to specific nectar components depend on their life style. AAs are a chemical EFN component that likely can shape the structure of ant–plant mutualisms.

Keywords *Acacia* · Ant–plant interaction · *Pseudomyrmex* · Mutualism · Nectar

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Introduction

Nectar is an aqueous solution of substances that mainly comprise primary metabolites such as sugars and amino acids and generally serves the attraction of mutualistic animals to plants (Baker and Baker 1975; Baker et al. 1978). Resulting benefits for plants include pollination in the case of floral nectar and protection from herbivores through the attraction of carnivores in the case of extrafloral nectar (EFN; Koptur 1992; Heil 2007, 2008). EFN is usually secreted outside the flowers, and—in contrast to floral nectar—it is not involved in pollination (Bentley 1977; Koptur 1992).

EFN has been described for plants in more than 300 genera (Bentley 1977; Koptur 1992; see also URL: www.biosci.unl.edu/emeriti/keeler/extrafloral/worldlistfamilies.htm). Plant species secreting EFN to attract defending ants (De la Fuente and Marquis 1999; Heil and McKey 2003) are, therefore, commonly called 'myrmecophilic' (i.e. 'ant-loving'). Ants benefit from attending plants, since they use EFN as a nutritive resource and since they are guided to

herbivores in the case of herbivore-induced EFN flow. Compounds that are mainly regarded responsible for the attraction of ants are sugars (Baker and Baker 1973; Blüthgen and Fiedler 2004; Heil et al. 2005) and amino acids (Lanza 1988, 1991; Lanza et al. 1993; Blüthgen and Fiedler 2004). Ants generally appear to prefer sugar solutions that contain amino acids over pure sugar solutions (Lanza 1991), but even the detailed identity of amino acids could elicit varying ant responses to artificial EFNs (Blüthgen and Fiedler 2004).

Other plants, in contrast, secrete EFN in a slightly different functional context. Obligate myrmecophytes are inhabited by specialised ants (Heil and McKey 2003). In these cases, there is no need for the plant hosts to attract ants from the vicinity, while the nutritive value of EFN might be more important due to the dependency of the inhabiting ants on the host-derived food rewards. A comparative approach using a set of related ant–plants that are characterised by these different levels of specificity allows, thus, a deeper understanding of the function that certain EFN components might play in ant attraction and nutrition.

For the present study, we used Mesoamerican *Acacia* species. In this species group, the obligate myrmecophytes secrete EFN constitutively (Heil et al. 2004) to nourish symbiotic ant colonies. These ants, which belong to the *P. ferrugineus* group of the genus *Pseudomyrmex*, obligatorily inhabit particular host plants and are nutritionally adapted to (and entirely dependent on) the host-derived food sources (Heil et al. 2004, 2005; Clement et al. 2008; Kautz et al. 2009). In contrast, non-myrmecophyte species of *Acacia* and of the related genera secrete EFN only in response to herbivore attack (Heil et al. 2004) to attract non-symbiotic ants, i.e. generalist species from the vicinity (Heil and McKey 2003). Thus, although EFN of both myrmecophyte and non-myrmecophyte *Acacia* species fulfils nutritive functions, an attractive function appears important only for the non-myrmecophytes, while EFN of myrmecophytes has likely a higher nutritional importance for the symbiotic ants.

The aim of our study was to evaluate how soluble amino acids affect ant preferences. We predicted that both sugars and amino acids should differ among EFN of myrmecophyte and non-myrmecophyte plant species. Both compound classes were analysed by high-performance liquid chromatography (HPLC), and then, behavioural assays were applied to study the attractiveness of nectar mimics differing in the quantity and concentration of those single compounds that most strongly contributed to the chemical differences among EFNs. Thereby, we aimed to determine whether specific amino acids, their concentration, or their mere number have any specific function in shaping different types of ant-plant mutualisms.

Methods and Materials

Plant Material and Study Site

We investigated the chemical composition of EFN of two myrmecophytes [*Acacia hindsii* Benth. and *Acacia cornigera* (L.) Willendow], of one non-myrmecophyte *Acacia* [*Acacia farnesiana* (L.) Willendow] and of one non-myrmecophytic, sympatric species of another genus, yet the same subfamily, the Mimosoideae (*Prosopis juliflora* Swartz). EFN was collected from plants growing naturally in the coastal area of the state of Oaxaca (México), 5 km northwest of Puerto Escondido (Pacific coast; ~15°55′ N and 97°09′ W; elevation, 15 m), in March and April 2007 and 2008. Species were determined following Janzen (1974) and Seigler and Ebinger (1995) and by comparison with specimens held at the Herbario MEXU at UNAM (Mexico City).

Nectar Collection and Quantification

Branches of myrmecophytes were deprived of ants and other insects the day before nectar collection: thorns were cut off, ants were mechanically removed, and the branch was then placed in a mesh bag after isolating it from the rest of the plant by applying a ring of sticky resin (Tangletrap, The Tanglefoot Corp., Grand Rapids, MI, USA). Branches of non-myrmecophyte species were induced by applying 1 mmol aqueous jasmonic acid solution (Heil et al. 2004) and then placed in mesh bags. After 1 day, nectar production rates were quantified as amounts of soluble solids per 24 h and per gram leaf dry mass by quantifying the nectar volume with microcapillaries (Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany) and the nectar concentration with a refractometer (Atago Co. Ltd.) as described previously (Heil et al. 2000, 2001). The leaves bearing the EFN were then collected and dried (50°C for 48 h). EFN was collected from five individuals per species, which, in earlier studies, (Heil et al. 2004, 2005) turned out a sufficiently high sample size to represent the chemical variability of EFN secretion in the populations under investigation.

Determination of Carbohydrates and Amino acids

EFN was stored at −20° until analysis. For carbohydrate analysis, 30 µL of nectar were diluted in 600 µL de-ionised water. After centrifugation and membrane filtration (Vivaspin 500, Vivascience Sartorius Group, Stonehous, UK), sugars were immediately separated by HPLC on an anion exchange column and quantified by pulsed amperometric detection (Dionex Series 4500 Chromatography System, Dionex, Idstein, Germany). For the analysis of amino acids,

30 µL of nectar were diluted in 200 µL de-ionised water. After centrifugation and membrane filtration, 100 µL of the supernatant were diluted with 20 µL sulfosalicylic acid (12.5%). After incubation at 4°C for 30 min and a second centrifugation, 50 µL of sample buffer were added to 100 µL of the supernatant. Samples were then analysed using an Amino Acid Analyzer LC 5001 (Biochrom 20 Plus, Cambridge, England). To control for differences in overall nectar concentration, the content of each amino acid was related to the sugar content of the respective sample and is expressed in millimole amino acid per millimole sugar. Differences in amino acid concentrations among the four species were evaluated with a Kruskal–Wallis analysis of variance (ANOVA) (N=5 individual per species). Different individuals were used as replicates to avoid pseudoreplication. Considering that data were not normally distributed, amino acid composition was evaluated with a non-metric multidimensional scaling (NMDS), in order to identify putative associations among the species (NMDS allows to reduce a multidimensional data set to two dimensions and thus appeared an appropriate approach for this question). Ordination was carried out using the following parameters: Bray–Curtis as distance measure, stability criterion of 0.005, 200 iterations, ten runs with real data and ten runs with randomised data. The software used for this analysis was PC-ORD v. 4.2 (McCune and Mefford 1999). Values of NMDS axes were compared among species using a univariate ANOVA.

Behavioural Assays

To study the behavioural responses of ants (symbiotic vs. non-symbiotic ants) to EFN with differing composition, 'cafeteria'-style experiments were carried out under field conditions. Such 'cafeteria' experiments allow to simultaneously offer different types of food sources to animals that freely can choose among them.

The NMDS of EFN amino acids revealed strongest differences between EFNs of *A. hindsii* and *Prosopis* (Fig. 3). We, therefore, focused on these two plant species for the behavioural assays and evaluated the attraction of obligate *Acacia* symbionts (*Pseudomyrmex ferrugineus* Smith F.) and of non-symbiotic ants to EFNs of these two plant species and to different artificial nectars that mimicked the major differences between the two plant species (see Table 1).

Experiment 1: High and Low AAs A first field eperiment was conducted in March 2007. EFN of *A. hindsii* and *Prosopis* was first collected from several individual plants (N=3–5) in the field and then pooled to achieve higher nectar volumes. Then, EFN collected of *A. hindsii* was adjusted with distilled water to a concentration of 4% (w/v), which was the highest concentration found in nectar of *Prosopis* in the field. Six nectar mimics were applied at the same concentration (4%): solution (sol.) 1 contained fructose (F)+glucose (G)+sucrose (S) at a ratio of 3:3:1 to mimick sugar ratio as found in the EFN of *Prosopis*, sol. 2 contained F+G at a ratio of 1:1, mimicking the sugars found in EFN of *A. hindsii*. Three nectar mimics were prepared with different AA compositions: Sol. 3 was a sugar solution (F/G=1:1) containing methionine, isoleucine, leucine, valine, threonine, phenylalanine, proline and serine (i.e., those AA that were highly correlated with Axis 1, see below, and that most strongly contributed to the chemical difference between EFN of *A. hindsii* and of *Prosopis*). Sol. 4 was a sugar solution (F/G=1:1) with those four AA that were highly dominant in EFN of *A. hindsii* (see below, Table 2), and sol. 5 was a sugar solution (F/G=1:1) containing phenylalanine and proline, which both appear particularly important AA in the physiology of insects (Chapman 1983; Dafni and Kevan 1994; Micheu et al. 2000). Pure water was offered as a control (sol. 6; Table 1).

These six artificial solutions and fresh EFNs of *A. hindsii* (sol. 7) and *Prosopis* (sol. 8) were offered to ants in their natural habitat. Two different AA/sugar ratios were used to evaluate whether ants are able to distinguish among different artificial solutions when these contain different AA/sugar ratios: (1) a ratio of each amino acid to fructose and glucose of 1:50 ('high-AA EFNs', N=10 cafeterias) and (2) a ratio of each amino acid to fructose and glucose of 1:1,000 ('low-AA-EFNs', N=17 cafeterias). The ratio 1:50 represents the values that we found in EFN of *Acacia* species (see Table 2).

Independent experiments were conducted for symbiotic and non-symbiotic ants. For *P. ferrugineus*, a 10-µl drop of each of the eight solutions was offered on a horizontal branch of an *A. hindsii* host plant (one cafeteria per plant). For generalist ants, the eight solutions were offered on branches of *Prosopis* that were cut off the plants and placed then on the soil to facilitate the access of generalist ants. In both cases, the individual droplets were offered 10–15 cm apart from each other, and the spatial order varied among the cafeterias. Solutions that had evaporated or that had been entirely consumed were replaced with a new drop of 10 µL. All ants feeding on the droplets were counted five times during the morning (between 10:00 A.M. and 13:00 P.M.). Each single count lasted 3 min, with an interval of 30–40 min between the individual censuses. Because ant abundance may differ among individual plants, numbers of ants that had been attracted to the individual cafeterias were summed up for every cafeteria to calculate the relative proportion of ants that had been attracted to each individual solution. This percentage of ants was subjected to univariate ANOVA (independent variable, solution

Table 1 Composition of amino acid solutions used for the "cafeteria experiments"

Substances	Sol. 1 F+G+S	Sol. 2 F+G	Sol. 3 F+G +8AA	Sol. 4 F+G +4AA	Sol. 5 F+G +2 AA	Sol. 6 Water	Sol. 7 *A. hindsii*	Sol. 8 *Prosopis*
Fructose	x	x	x	x	x			
Glucose	x	x	x	x	x			
Sucrose	x		x					
Isoleucine			x					
Leucine			x	x				
Methionine			x					
Phenylalanine			x	x	x			
Proline			x	x	x			
Serine			x					
Threonine			x					
Valine			x	x				
Pure water						x		
EFN *A. hindsii*							x	
EFN *Prosopis*								x

8AA, 4AA and 2AA mean the addition of the respective amino acids as shown in the table to the F (fructose)+G (glucose) sugar solution

type) after arcsine transformation (Sokal and Rohlf 1995). A least significant difference (LSD) test was posteriorly applied.

Experiment 2: Number of AAs A second 'cafeteria experiment' was carried out in January 2009 to examine whether the ratio of AAs to sugars or the number of AAs is most important to determine ant preferences. Given that ants were only able to distinguish among solutions at higher AA concentrations (Fig. 4), solutions (4%) at ratios 1:10 and 1:50 of AAs to total sugars were prepared with different numbers of total AAs (2AA, 4AA and 8AA). The following six solutions were prepared: 1:10-2AA, 1:10-4AA, 1:10-8AA, 1:50-2AA, 1:50-4AA and 1:50-8AA. Solutions were

Table 2 Concentration of single amino acids (AAs) (μmol L^{-1}), total AAs (mmol L^{-1}) and total sugars (mmol L^{-1}) in EFN of *A. cornigera*, *A. hindsii*, *A. farnesiana* and *Proposis juliflora*

	A. cornigera	*A. hindsii*	*A. farnesiana*	*Prosopis*
ALA (**)	1,846±336	924±102	364±93	178±51
ARG (**)	0±0	10±10	24±14	280±152
ASN (*)	3,375±187	581±237	7,120±2,187	1275±605
ASP (**)	176±15	335±79	496±126	963±355
GLN (**)	1,186±170	831±449	1,473±408	206±121
GLU (***)	1,922±138	2,441±848	302±46	294±27
GLY (*)	86±10	209±46	256±32	196±65
HIS (**)	2,770±359	1,595±158	278±93	469±62
ILE (***)	857±139	1,808±207	285±125	7±5
LEU (***)	1,405±196	3,462±285	56±22	22±7
LYS (ns)	40±17	46±12	38±10	74±21
MET (***)	400±94	1,148±93	44±21	0±0
PHE (***)	13,127±2,672	12,738±2,085	2,809±527	2,066±150
PRO (***)	1,238±205	912±364	195±96	0±0
THR (*)	450±32	805±83	498±123	124±23
TRP (**)	1,489±399	339±86	452±92	938±158
TYR (*)	4,606±477	1,533±155	1,484±300	4,816±469
SER (**)	941±141	1,001±262	1,368±196	381±94
VAL (***)	1,712±196	4,281±468	620±191	165±36
Total AAs	37±0.6	34±0.6	18±0.3	12±0.2
Total Sugars	827±118	336±34	356±44	562±62

Statistical differences among the four species were evaluated for each AA with a Kruskal–Wallis ANOVA, and significance levels are indicated. For amino acid names, see Table 3. Total AAs refers to the sum of the 19 AAs for each species. Total sugars refer to the sum of fructose and glucose for *A. cornigera* and *A. hindsii* and of fructose, glucose and sucrose for *A. farnesiana* and *Prosopis* (see Fig. 1).

ns $P>0.05$,*$P<0.05$, **$P<0.01$, ***$P<0.001$

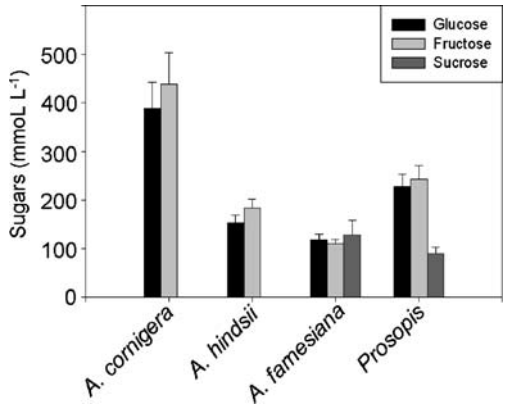

Fig. 1 Sugar quantities in EFN. Concentrations are depicted in mmol sugars per L EFN as means±SE. Sample size N=5 individuals per species

offered in independent experiments to symbiotic (N=10) and non-symbiotic ants (N=10). 'Cafeteria experiments' were conducted as described above. Differences in the percentage of ants attracted to each solution were analysed with univariate ANOVA, after arcsin transformation. LSD was applied then as post hoc test.

Experiment 3: AA/sugar ratios The third experiment was conducted January 2009 to determine which minimal ratio of AAs to total sugars allows ants to differentiate among mimics that contain and that do not contain AAs. Six different 4AA solutions (4%) for symbiotic ants (N=10) and six different 8AA solutions (4%) for non-symbiotic ants (N=10) were prepared at different ratios of AAs to total sugars (1:10, 1:50, 1:100, 1:500 and 1:1,000) and tested in 'cafeteria experiments'. Differences in ant preferences (percentage of ants) among solutions were analysed with univariate ANOVA, after arcsin transformation. A LSD test was posteriorly applied. 'Cafeteria experiments' were conducted as described above.

Results

Sugars and Amino Acids

Sucrose, fructose and glucose were the only sugars detected in EFN of *Acacia* and of the closely related *Prosopis*. EFNs of the two non-myrmecophyte species contained all three sugars, while EFNs of the myrmecophytes only contained the monosaccharides, fructose and glucose (Fig. 1). EFN secretion (in microgram soluble solids per gram leaf dry mass per 24 h) by the myrmecophyte, *A. cornigera*, was significantly higher than for the non-myrmecophyte species ($F_{3,21}$=6.08; P<0.005; univariate ANOVA; Fig. 2). No significant differences were observed in EFN secretion between *A. cornigera* and *A. hindsii* (P>0.05, Tukey test) and between *A. hindsii* and the non-myrmecophyte species (P>0.05, Tukey test).

Amino acid concentrations varied strongly among the four species, and 'species' was a significant source of variation in the concentrations of 17 of the 19 amino acids detected (Table 2). The qualitative compositions differed much less, as only two of the four species contained less than 19 amino acids (*A. cornigera*, arginine missing; *Prosopis*, methionine and proline missing), while in EFN of *A. hindsii* and *A. farnesiana*, all the 19 amino acids were present.

Non-metric Multidimensional Scaling

Both axes contributed significantly to the variation among the species (axis 1, $F_{3,16}$=63.0, P<0.001, univariate ANOVA; axis 2, $F_{3,16}$=22.4, P<0.001, univariate ANOVA), allowing a grouping of myrmecophyte vs. non-myrmecophyte species. *A. hindsii* and *Prosopis* were most distant from each other (Fig. 3). For axis 1, there were no significant differences among myrmecophyte species and among non-myrmecophytes, but the myrmecophtes differed significantly from the non-myrmecophytes. For axis 2, *Prosopis* was significantly different from all other three species.

Methionine, isoleucine, leucine, valine, threonine, phenylalanine, proline and serine were the components with the highest contribution to both axes (amino acids with higher correlation coefficients, see Table 3), suggesting that these eight amino acids did increase the C value and thus contributed most strongly to the differentiation among the species. All these eight amino acids where present at much higher concentrations in *A. hindsii* EFN than in EFN of *Prosopis* (Table 2).

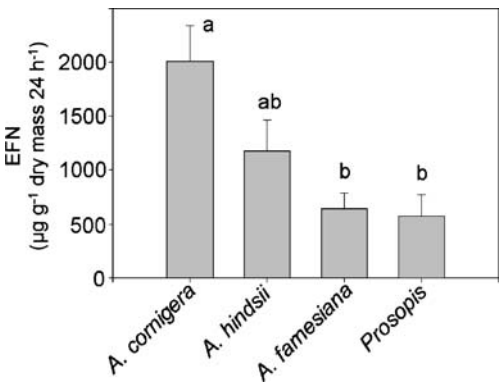

Fig. 2 EFN secretion rates. Amounts of total soluble solids (µg secreted per g leaf dry mass and per 24 h) are depicted for *A. cornigera*, *A. hindsii*, *A. farnesiana* and *Prosopis* as means±SE. Sample size N=5 individuals. *Different letters* indicate significant differences (P<0.05 according to post hoc Tukey test) among the species

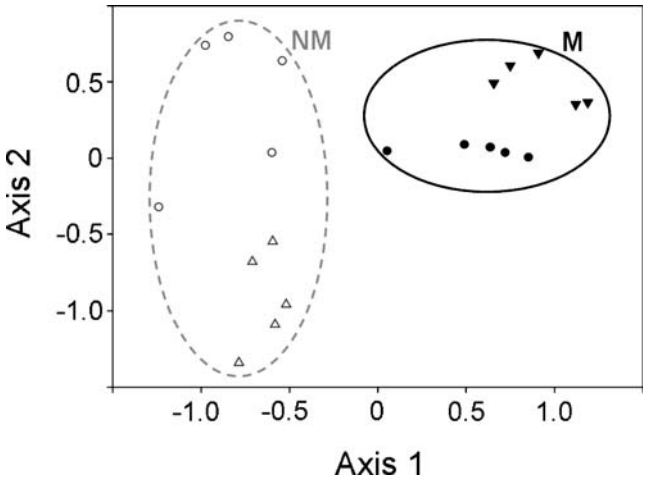

Fig. 3 Non-metric multidimensional scaling (NMDS) ordination diagram of amino acid composition in EFNs. *Black circles*, *A. cornigera*; *black triangles*, *A. hindsii*; *white circles*, *A. farnesiana*; *white triangles*, *Prosopis*; *M* myrmecophytes; *NM* non-myrmecophytic species

Amino Acids and Ant Attraction

In the experiment using low AA EFNs (ratio of each AA to fructose=1:1,000 in the artificial mimics), mutualistic ants preferred EFN of *A. hindsii* over EFN of *Prosopis* (Fig. 4a), whereas non-mutualistic ants showed the opposite preference (Fig. 4b). In general, 'solution type' significantly affected the percentage of ants attracted to the different solutions. This remained true both for symbiotic ants ($F_{7,128}$=8.31; P<0.001; univariate ANOVA) and for non-symbiotic ants ($F_{7,128}$=7.49; P<0.001; univariate ANOVA). Nevertheless, neither symbiotic nor non-symbiotic ants discriminated among the various AA-containing artificial solutions (Fig. 4a, b). For high AA EFNs, the percentages of ants attracted to the different solution types also were significantly different both for symbiotic ants ($F_{7,72}$=10.89; P<0.001; univariate ANOVA) and non-symbiotic ants ($F_{7,72}$=10.83; P<0.001; univariate ANOVA; Fig. 4c, d). Moreover, ants under these conditions distinguished among the artificial solutions, since symbiotic ants significantly preferred the artificial solution with four amino acids (leucine, phenylalanine, proline and valine), while no significant differences were observed among the other artificial solutions. Again, symbiotic ants preferred EFN of *A. hindsii* over the EFN of *Prosopis* (Fig. 4c). On the other hand, non-symbiotic ants significantly preferred the sugar solutions with sucrose over the solution without sucrose, and the sugar–amino acid solutions over sugar-only solutions, although they did not discriminate among the different solutions with amino acids. Consistently with the first experiment, *Prosopis* EFN attracted more non-symbiotic ants than nectar of *A. hindsii* (Fig. 4d).

In the second experiment testing different AA/sugar ratios, significant differences among AA solutions were only observed for symbiotic ants ($F_{5,54}$=6.66; P<0.001; univariate ANOVA). These ants significantly preferred the solution 1:10 over all other solutions, and in fact, ant preference decreased continuously with decreasing AA concentration (Fig. 5a). In contrast, non-symbiotic did not differentiate significantly among solutions with different AA/sugar ratios ($F_{5,54}$=0.27; P>0.05; univariate ANOVA; Fig. 5b). Similar results were obtained in the third experiment, where symbiotic ants distinguished among the different solutions ($F_{5,54}$=0.47; P>0.05; univariate ANOVA, see Fig. 5a) and significantly preferred the solution with 4AAs over the other solutions at both 1:10 and 1:50 ratios ($F_{5,54}$=4.67; P<0.001; univariate ANOVA, see Fig. 5c). Again, non-symbiotic ants did not differentiate significantly among solutions ($F_{5,54}$=0.27; P>0.05; univariate ANOVA; Fig. 5d)

Discussion

EFN is secreted by many plants to attract ants and thus serves as a means of indirect defence (Heil 2008). Sugars and amino acids are generally known as the EFN compounds that are responsible for this attractive function (Koptur 1979; Lanza and Krauss 1984; Lanza 1991; Lanza

Table 3 Correlations between specific amino acids and the two NMDS axes in EFN of three *Acacia* species and *Prosopis*

	NMS I	NMS II
Amino Acids		
ALA (alanine)	0.64	0.31
ARG (arginine)	−0.32	−0.38
ASN (asparagine)	−0.36	0.45
ASP (aspartic acid)	−0.39	−0.33
GLN (glutamine)	0.08	0.66
GLU (glutamic acid)	0.70	0.40
GLY (glycine)	−0.32	0.23
HIS (histidine)	0.81	0.24
ILE (isoleucine)	0.88	0.66
LEU (leucine)	0.91	0.49
LYS (lysine)	−0.14	−0.18
MET (methionine)	0.87	0.51
PHE (phenylalanine)	0.89	0.35
PRO (proline)	0.67	0.37
THR (threonine)	0.61	0.82
TRP (tryptophan)	0.18	−0.26
TYR (tyrosine)	0.02	−0.65
SER (serine)	0.37	0.74
VAL (valine)	0.87	0.56

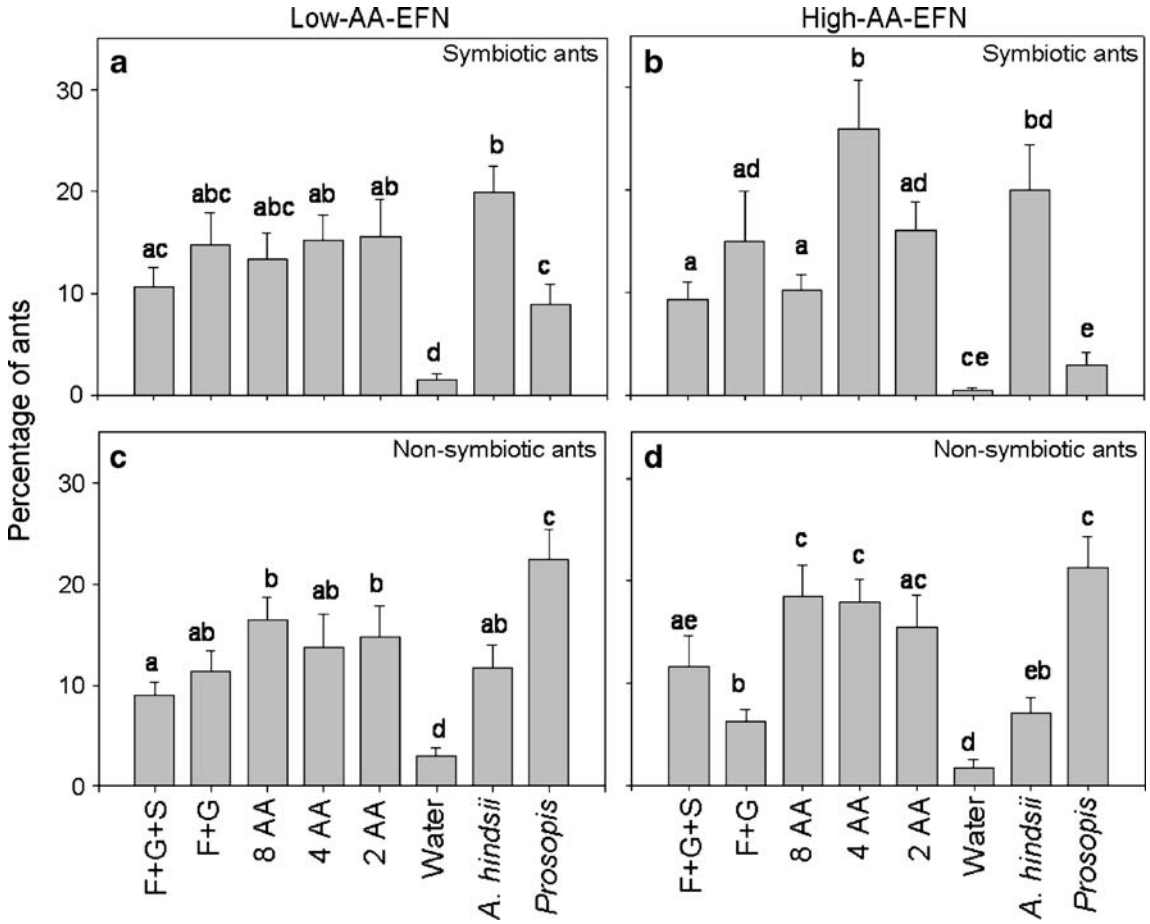

Fig. 4 Ant attraction to EFN mimics. Preferences of symbiotic and non-symbiotic ants to natural EFNs of *A. hindsii* and *Prosopis* and various EFN mimics with and without amino acids (AA). Solution compositions are indicated in Table 1. Low-AA-EFN (**a**, **b**) contained an AA/sugar ratio of 1:1,000 (sample size=17 cafeterias), whereas high AA EFNs (**c**, **d**) contained a ratio of 1:50 (sample size=10 cafeterias). Ant preferences are expressed as means+SE of the percentage of all feeding ants that were attracted to each solution. *Different letters* indicate significant difference in ant attracted among solutions ($P<0.05$ according to post hoc LSD test)

et al. 1993; Blüthgen et al. 2004), but ant preferences to sugars and amino acids may vary among ant species according to their nutritive needs and particularly among functionally different types of mutualisms.

NMDS analysis demonstrated a separation of myrmecophyte species vs. non-myrmecophytes according to the amino acid composition of their EFN: the myrmecophyte, *A. hindsii*, and the non-myrmecophyte, *P. juliflora*, turned out to be the most distant among the four investigated species. Interestingly, these chemical distances mirror the phylogenetic relations: a phylogenetic reconstruction based on chloroplast DNA markers (Heil et al. 2004) also revealed *A. hindsii* and *P. juliflora* to be most distantly related among the species tested in this study. Phenylalanine and proline appeared in much higher concentrations in EFN of the myrmecophytes, *A. cornigera* and *A. hindsii*, than in EFN of the two non-myrmecophytes, which is in line with the very low concentrations of these two amino acids found in EFN of the non-myrmecophyte, *Macaranga tanarius* (Heil et al. 2000) and in other EFNs (Baker et al. 1978; Inouye and Inouye 1980). These two amino acids were among those that most intensively contributed to the differentiation that NMDS revealed among the EFNs studied in this paper.

We found that free amino acids at high concentrations in EFN significantly affected preferences by different ants. Several studies have reported interspecific variability in ant preferences to amino acids (Lanza 1988; Lanza et al. 1993; Blüthgen and Fiedler 2004). Our results generally confirm these studies, since symbiotic and non-symbiotic ants differed in their preferences for artificial amino acid solutions. Nevertheless, differences in ant behaviour were only evident when the relative concentration of single amino acids to sugars were high (1:50), i.e., at concentrations as found in *Acacia* EFN. In contrast, neither symbiotic nor non-symbiotic ants discriminated among artificial mixtures at low amino acid concentrations (1:1,000). This result confirms the study by Lanza (1991), who showed that preferences of fire ants were most obvious when nectar mimics contained high levels of amino acids.

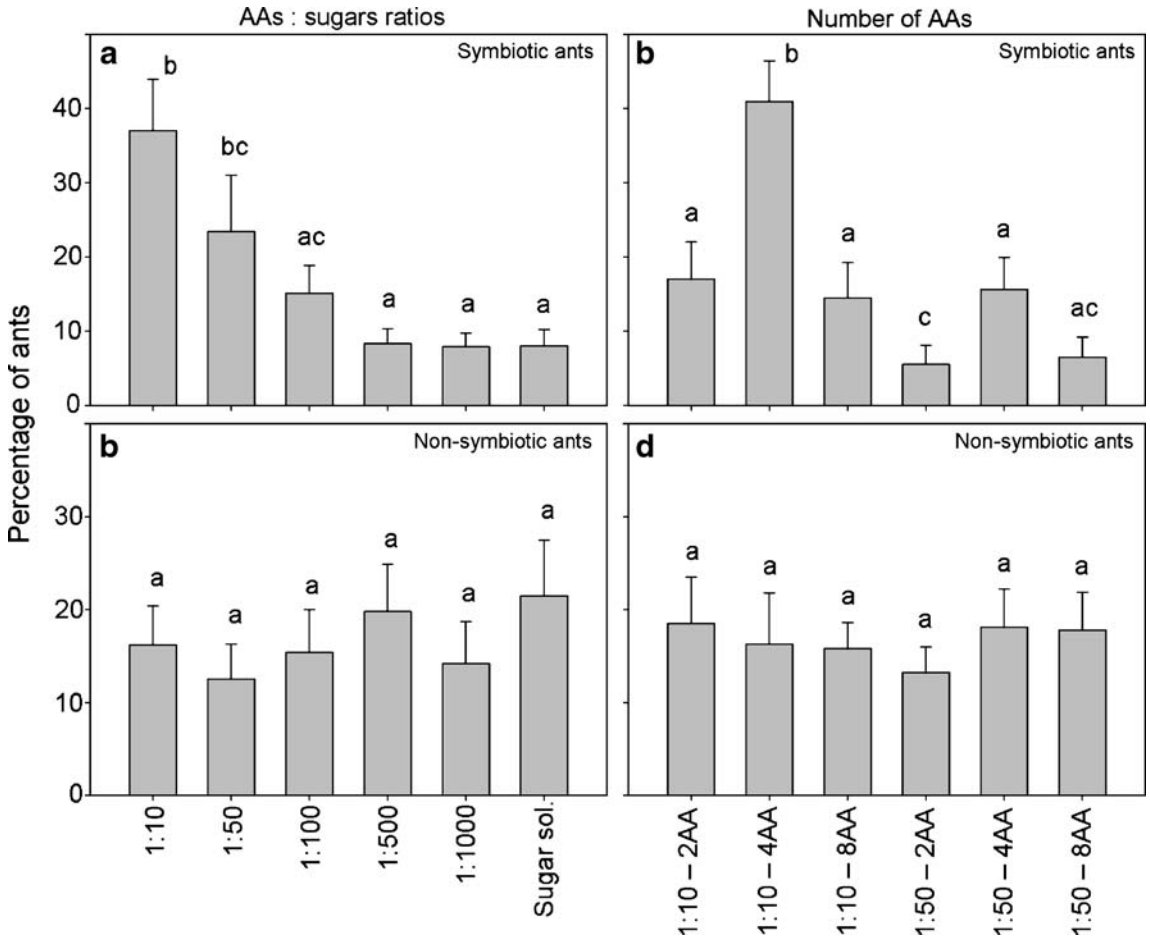

Fig. 5 Preferences of symbiotic and non-symbiotic ants to 4AA and 8AA solutions, respectively, with different AA/sugar ratios (**a**, **b**) (sample size=10 cafeterias) and to solutions with different number of AAs at two different AAs/sugars ratios (**c**, **d**) (sample size=10 cafeterias). Ant preferences are expressed as means+SE of the percentage of all feeding ants that were attracted to each solution. *Different letters* indicate significant difference in ant attracted among solutions ($P<0.05$ according to post hoc LSD test)

Therefore, our results support the general assumption that high concentrations of amino acids in nectar contribute notably to its taste (Gardener and Gillman 2002).

However, ant life history strongly affected whether and how ants responded to certain nectar components and AAs that affected the chemical grouping of myrmecophyte EFNs vs. non-myrmecophyte EFNs determined in part the observed behaviour of symbiotic and non-symbiotic ants. As expected, the symbiotic ants specifically preferred the solution containing those four amino acids that are highly concentrated in the EFN of their host plant (*A. hindsii*). Furthermore, symbiotic ants were able to distinguish this specific solution (1:10-4AA) from other solutions, suggesting that not only AA concentration but also their number and detailed identity determines preferences by symbiotic ants. In contrast, although non-symbiotic ants preferred the solution with eight amino acids in the first experiment, they did not distinguish among nectar mimics that differed only in the number or exact concentration of AAs, while the identity of sugars had a strong and significant effect. Apparently, for generalist ants, just the presence of amino acids in the nectar but not their detailed identity is important, while symbiotic ants are much more selective. Considering that non-symbiotic ants do not establish an obligate mutualism with plants, they must forage on different plant species, unlike symbiotic ants, which are constitutively nourished by one specific host. This different style of life between symbiotic and non-symbiotic ants affects their preferences and selectiveness with respect to detailed chemical composition of their food sources.

These results also suggest that those 4AAs that contributed most to separate myrmecophyte from non-myrmecophyte EFN and that significantly affected the behaviour of symbiotic ants should have a particularly important function for the nutrition of these ants. In fact, high concentrations of phenylalanine and proline have also been reported for different floral nectars (Carter et al. 2006; Petanidou et al. 2006) and thus might be typical for more important types of

nectar-mediated interactions. Phenylalanine is one of the ten essential amino acids for honeybees (Chapman 1983; Dafni and Kevan 1994), while proline is preferentially utilised by insect pollinators during the initial phases of insect flight (Micheu et al. 2000). For ants, comparable information is lacking, and further physiological studies are needed to determine the significance of specific essential amino acids for their metabolism.

Our study suggests that the detailed amino acid composition of EFN is important for its ecological function. Strikingly, behavioural responses differed between symbiotic ants and non-symbiotic ants, with symbiotic ants generally being more selective. Our results confirm that both amino acids and sugars contribute to the taste and attractiveness of nectars and demonstrate clearly that the responses of ants to specific nectar components depend on their life style. Therefore, amino acids are a chemical component of nectar that likely can shape the structure of ant–plant mutualisms.

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Effects of Simulated Herbivory on Defensive Compounds in Forage Plants of Norwegian Alpine Rangelands

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Abstract A field study on the effects of current grazing practices on plants in central Norway found no increase in either phenolic compounds or proteinase inhibitors in plants subjected to grazing by sheep. This could either reflect insufficient damage to the plants due to low grazing intensity or a lack of a long-term response of the plants to grazing. In this study, we tested the hypothesis that damage to forage plants used by sheep and rodents in Norwegian alpine rangelands can stimulate a long-term (at least 2-week) increase in levels of defensive compounds. We used clipping experiments to manipulate the severity and timing of damage to eight species of common plants used by herbivores in Norway. Under greenhouse conditions (i.e., climate-controlled), we subjected mature plants to one of four clipping treatments: control (0% leaf tissue removed), low (10–15% leaf tissue removed), high (70–75% leaf tissue removed), or sustained (15% of leaf tissue removed every other day up to a total removal of 75%, i.e., five clippings over 9 days). Samples were collected 2 weeks after final clipping and analyzed for concentrations of total phenolics, proteinase inhibitors, ratio of total phenolics to soluble proteins, and ratio of proteinase inhibitors to soluble plant proteins. As expected, the different species of plants responded differently to simulated herbivory, but most plants either showed no response to mechanical wounding and tissue loss or had reduced defensive compounds. Thus, our results do not support the hypothesis that herbivory induces a long-term increase in defensive compounds in alpine rangelands of Norway, a result consistent with those from field studies.

Keywords Alpine plants · Induced defensive compounds · Proteinase inhibitors · Simulated herbivory · Total phenolics

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Introduction

Plants often increase levels of secondary metabolites in response to herbivory (an induced response), but responses to defoliation and browsing vary depending on nutrient availability, season and severity of the attack, species of plant, and species of herbivore (Crawley 1999). The production of defensive compounds constitutes a significant energetic cost to the plant and, therefore, may reduce seed production (Cates 1975) or lower growth rates (Zangerl et al. 1997; Donaldson et al. 2005). Such plants would be at a disadvantage when levels of herbivory are low, but habitats with variable levels of herbivory may favor plants that can increase their level of defense as herbivory increases (Adler and Karban 1994; Åström and Lundberg 1994; Müller-Schwarze and Thoss 2008). Indeed, induced responses often increase plant fitness (Agrawal 1998), which indicates that the benefits of induced defenses often outweigh the cost of producing defensive chemicals.

Induced responses occur in response to a wide range of herbivores, including mammals. For instance, severe winter browsing by hares causes feltleaf willow to revert to a juvenile phase that contains much higher levels of phenolic compounds (Bryant et al. 1985). Similarly, grazing by

livestock (cattle, sheep, and goats) stimulates production of leaf-surface terpenes in tarbush (Estell et al. 1998) and grazing by Soay sheep increases alkaloid levels in grasses on the island of St. Kilda (Bazely et al. 1997). Although many plants respond to herbivory with increased defensive compounds, some do not. Bilberry (*Vaccinium myrtillus*) in northern Norway lowers phenolic concentrations when heavily used by grey-sided voles (Oksanen et al. 1987). In other cases, the data do not agree. For instance, Seldal et al. (1994) report increased production of proteinase inhibitors in response to clipping for stiff sedge (*Carex bigelowii*) in Norway, but Lindgren et al. (2007) found no such increase from an extensive clipping experiment in northern Sweden.

Sheep and arvicoline rodents (lemmings and voles) occur together on Norwegian alpine rangelands where both forage mainly on graminoids and forbs (Mysterud and Warren 1991; Mysterud and Mysterud 2000; Mysterud and Austrheim 2005; Kausrud et al. 2006; Saetnan et al. 2009). Thus, sheep can potentially impact the quality or quantity of available forage for the rodents. Högstedt and Seldal (1998) suggest that grazing by even low densities of sheep (12/km^2) has detrimental effects on small herbivores because of lower forage quality. Arvicoline rodents do appear sensitive to changing levels of defensive compounds in preferred forage plants. Lemmings prefer plants low in proteinase inhibitors and with a low ratio of proteinase inhibitors to plant proteins (Bråthen et al. 2004), while voles prefer forage with lower phenolic concentrations (Hambäck et al. 2002; Strengbom et al. 2003).

A field study on the effects of current grazing practices on plants and rodents in central Norway reports no increase in either phenolic compounds or proteinase inhibitors in plants (including *C. bigelowii*) subjected to grazing by sheep (Saetnan 2008). This could reflect either insufficient damage to the plants or a lack of a long-term plant response to grazing. Both of the studies cited above report short-term, transient responses (hours to days) to *C. bigelowii* herbivory (Seldal et al. 1994; Lindgren et al. 2007). For increased defensive compounds to inhibit future grazing, higher concentrations must be maintained over a longer period, and maintenance of higher levels may require repeated damage (Gustafson and Ryan 1976), e.g., every 1–3 days if only transient responses occur.

This study tested the hypothesis that damage to forage plants used by sheep and rodents in Norwegian alpine rangelands can stimulate long-term (at least 2-week) increases in defensive compound levels. Plants contain a wide variety of defensive compounds that could be induced (Rosenthal and Berenbaum 1991), too many to include in one study. In this study, we concentrated on phenolic compounds and proteinase inhibitors, two kinds of compounds induced by herbivory in many plants. The test involved clipping experiments that manipulated the amount and repetition of damage to eight species of common plants used by herbivores in central Norway. We expected increased concentrations of phenolic compounds and proteinase inhibitors or changes in the ratios of these compounds to soluble plant proteins in response to high or sustained levels of damage.

Methods and Materials

Choice of Plants

Although sheep eat mainly graminoids and forbs, they also forage regularly on young shrubs, such as bilberry and willow (Mysterud and Warren 1991; Mysterud and Mysterud 2000; Mysterud and Austrheim 2005; Kausrud et al. 2006). The only detailed study of the diet of sheep in alpine rangelands provides a representative diet for similar habitats across southern and central Norway (Mysterud and Austrheim 2005) and forms the basis for the choice of plants used in this study. The dominant graminoids in alpine rangeland sheep diets include wavy hair grass (*Deschampsia flexuosa*), stiff sedge (*C. bigelowii*), and sweet vernal grass (*Anthoxanthum odoratum*). In addition, tufted hair grass (*Deschampsia cespitosa*), which dominates the meadow habitats of central Norway, provides a sizeable proportion of the diet. The most common species of forbs in sheep diets are common sorrel (*Rumex acetosa*) and wood cranesbill (*Geranium sylvaticum*). Goldenrod (*Solidago virgaurea*), though eaten in small amounts, occurs in a far larger proportion of the diet than its availability suggests. To complete the study, we included bilberry (*V. myrtillus*) as a representative of the dwarf shrubs eaten by sheep. Field voles (*Microtus agrestis*) in central Norway also eat a diverse diet of graminoids, forbs, and shrubs and overlap with sheep in several of their preferred species (Table 1). Norwegian lemmings primarily eat graminoids and mosses,

Table 1 Relative use of selected forage plants as indicated by occurrence in the diet of sheep, lemmings, and voles

Plant species	Sheep[a]	Lemmings[b]	Voles[b]
Carex bigelowii	++	+++	++
Anthoxantum odoratum	++	+	+
Deschampsia cespitosa	+	++	++
Deschampsia flexuosa	+++	–	+
Geranium sylvaticum	++	+	+
Solidago virgaurea	+	–	+
Rumex acetosa	++	–	+
Vaccinium myrtillus	+	+	++

– not eaten, + <5% of diet, ++ 5–15% of diet, +++ >15% of diet

[a] Data for sheep from Mysterud and Austrheim (2005)

[b] Data for lemmings and voles from Saetnan et al. (2009)

including significant amounts of stiff sedge and tufted hair grass (Table 1).

Methods of Propagation

Because this experiment was conducted in a greenhouse at the University of Essex, England, we procured plants from commercial nurseries that specialize in wild plants. The only exception, *C. bigelowii*, was collected from the southerly slope of the Carneddau ridge in North Wales, a site identified by Britton et al. (2005). All plants were maintained in a greenhouse for a minimum of 1 month before the start of clipping trials, generally grew to triple their original height, and were flowering at the time of clipping. No signs of nutrient deficiency, such as slow growth or discoloration of tissue, occurred.

Upon accession, we transplanted all plants into plastic pots appropriate for their size, 0.5-L pots for *Carex*; 2-L pots for the three grasses, *R. acetosa* and *S. virgaurea*, and 5-L pots for *V. myrtillus* and *G. sylvaticum*. All pots contained Vapogro™ peat-based potting compost (Avoncrop, UK) and were kept well-watered throughout the study. No further nutrients were added. We maintained plants at 10–15°C with 20 h of daylight, cycling between warmer daytime and cooler nighttime temperatures, which simulated summer conditions in the alpine regions of central Norway.

Experimental Regime

Previous investigations of grazing pressure in alpine habitats of southern Norway showed that low densities of sheep (25/km^2) damaged 3–9% of forage plants, except for goldenrod, which had 25–43% of plants damaged. High densities of sheep (80/km^2) damaged 10–23% of plants overall, but 74% of goldenrods (Mysterud and Austrheim 2005). A previous clipping study of stiff sedge removed 10–15% of leaf tissue to simulate low-intensity herbivory and 60–70% to simulate high intensity of herbivory (Lindgren et al. 2007). Seldal et al. (1994) used a single clipping of stiff sedge with removal of 50% of leaves. To examine the potential impact of different levels of grazing on defense compounds, we compared four levels of clipping: control (0% removed), low (10–15% of all shoots removed), high (70–75% of all shoots removed), and a sustained high treatment (15% of all shoots removed every other day up to a total removal of 75%, i.e., five clippings over 9 days). Clipping consisted of trimming terminal portions of all shoots the required amount with a clean scissors, including leaves, stems, and flowers (if any). In all cases, samples (similar amounts of old and regrown leaves) were harvested from each of the six replicates for each treatment and species combination (a total of $6 \times 4 \times 8 = 192$ samples for each chemical test) 2 weeks after the final experimental clipping.

Chemical Analysis

All samples were frozen in liquid nitrogen immediately upon collection and ground to a fine powder using a mortar and pestle. Samples for total phenolic (TP) analysis were extracted in 80% aqueous methanol for 24 h, centrifuged, and the supernatant analyzed by using the Folin–Ciocalteau assay with a gallic acid standard (Singleton et al. 1999). To analyze for proteinase inhibitors (PI), a 10-mg sample was extracted in extraction buffer (1 mL, 40 mM Tris–HCL buffer, pH 8.1, with 2% *w*/*v* Triton X-100). Extracts were measured for PI activity by using a standard assay with trypsin (Bergmeyer and Gawehn 1974), so we may have only assayed trypsin inhibitors. Because previous work indicated lemmings may respond to the ratio of PI to soluble plant proteins (SPP), not simply PI (Seldal et al. 1994), we also assayed SPP, which we quantified by using a protein dye-binding method (Bradford 1976) with bovine serum albumin (Sigma) as the standard. We then calculated the ratio of PI to SPP for each sample and the ratio of TP to SPP. Because phenolic compounds often bind to proteins (Harborne 1991), we also calculated the ratio of TP to SPP.

Statistical Analysis

To test overall significance of treatments, results for all chemicals were analyzed together by using a two-way multivariate analysis of variance (MANOVA) with species and treatment as fixed factors. Roy's largest root was used for tests of overall significance because it provided the greatest power and the most robust test statistic for further post hoc tests (Scheiner 2001). After establishing overall significant patterns in the results, we analyzed differences among treatments, within species, by using a separate one-way MANOVA for each species with treatment as a fixed factor. We then tested mean values of specific chemicals for significant differences between treatments and between species with Tukey's post hoc tests. All statistical analyses were done with SPSS 14.0 (2005).

Results

The overall two-way MANOVA indicated that treatment effects ($F_{3,151}=12.1$, $P<0.001$), species effects ($F_{7,151}=1097.6$, $P<0.001$), and their interactions ($F_{21,151}=9.6$, $P<0.001$) were all significant for all chemicals with two exceptions. Neither the treatment effect for TP ($F_{3,151}=1.0$, $P=0.382$) nor the treatment effect for TP/SPP ($F_{3,151}=1.9$, $P=0.131$) were significant, although the species by treatment interactions were (TP: $F_{21,151}=8.3$, $P<0.001$; TP/SPP: $F_{21,151}=4.4$, $P<0.001$). Contrary to expectations, the control and low-intensity clipping treatments had higher

levels of proteinase inhibitors ($F_{3,151}$=15.8, P<0.001), but the species by treatment interaction ($F_{21,151}$=8.4, P<0.001) indicated that chemical responses to treatments differed among the plant species. The significant treatment effects for SPP and TI/SPP (SPP: $F_{3,151}$=7.5, P<0.001; TI/SPP: $F_{3,151}$=5.5, P=0.001) also differed from expectations being higher in the control and low-intensity clipping treatments, and again, the species by treatment interactions (SPP: $F_{21,151}$=2.7, P<0.001; TI/SPP: $F_{21,151}$=5.5, P=0.001) indicated different responses among species.

Within species, PI showed significant treatment effects only in *Solidago* and *Carex*, and these were not in the directions expected (Table 2). Compared to controls, PI increased in *Solidago* only at low clipping intensity and actually decreased in *Carex* at high and sustained levels of clipping.

Two species also showed a significant response to treatment in the concentration of SPP, although the overall treatment effect was also significant (Table 2). *D. flexuosa* had a lower SPP concentration in the sustained treatment than in the control, whereas *Rumex* had a higher SPP concentration in the high clipping treatment, but not in other treatments. *Vaccinium* had lower SPP at sustained clipping compared to high clipping, but neither treatment differed from the control.

The overall pattern for the ratio of PI to SPP was similar to that for PI, but in this case, five species showed a significant response to the treatments (Table 2). Compared to the controls, only *D. cespitosa* responded in the expected direction with an increased ratio, but only at high clipping intensity. *Solidago* and *Vaccinium* exhibited a higher ratio at low clipping intensity only, *Rumex* had a significantly lower ratio at high clipping intensity only (although low and sustained clipping produced the same trend), and *Carex* showed a lower ratio at high intensity and sustained clipping.

The significant species by treatment interaction for TP occurred because five species showed a variety of treatment effects (Table 2). Compared to the controls, two species could be regarded as responding in the expected direction, although the patterns were not consistent with one another. *Anthoxanthum* showed an increased TP at low and high intensities of clipping only, and *Geranium* had increased phenolics with sustained clipping only. Two species responded in the opposite direction. The TP content of *Rumex* decreased significantly at all levels of clipping, whereas that of *Vaccinium* decreased only with sustained clipping. The fifth species, *Carex*, had higher TP at high clipping than at sustained clipping, but neither differed from the control.

Similarly, the significant species by treatment interaction for TP/SPP occurred because three species showed varied treatment effects (Table 2). *Carex* had higher TP/SPP at high compared to sustained clipping, and *Rumex* had lower TP/SPP at all levels of clipping compared to the control, both mirroring the patterns for TP. *D. flexuosa* had higher TP/SPP with sustained clipping because of lower SPP with this treatment.

Discussion

As expected, the different species of plants responded differently to simulated herbivory. In general, the forage plants used in our experiment showed little propensity for increased concentrations of defensive compounds or their ratio to SPP in response to damage. Out of 32 cases (2 defensive chemicals×2 ratios to SPP×8 species), four responses to treatments showed increased concentrations at high or sustained damage compared to controls (PI/SPP ratio for *D. cespitosa*, TP for *Anthoxanthum* and *Geranium*, and TP/SPP for *D. flexuosa*), six were in the opposite direction (PI for *Carex*, PI/SPP for *Rumex* and *Carex*, TP for *Rumex* and *Vaccinium*, and TP/SPP for *Rumex*), and the rest showed increased concentrations only at low clipping (three cases) or no significant difference from the controls (19 cases).

Other studies, such as for *Solidago missouriensis* (Brown and Weis 1995) and *Brassica napus* (Cipollini and Bergelson 2000), have shown induced responses of dicotyledons after little damage, but no response after serious damage. A lack of response to severe damage in such cases might reflect resource limitation or lack of tissue for provision of the necessary jasmonic acid signal (Cipollini and Sipe 2001). The response of PI and PI/SPP in *Solidago* to low clipping only in our study could be a similar phenomenon, but that still leaves a preponderance of cases with no response or a response opposite to that expected for our treatments.

Differences in resource availability in the laboratory and the field could also explain the previous contradictory results for PI in *Carex*, a transient increase reported by Seldal et al. (1994), but no response reported by Lindgren et al. (2007). However, our longer-term results (decreased PI only at high and sustained damage) differed from both these studies. Curiously, concentrations of PI in our *Carex* controls were four times those reported in the field by Bråthen et al. (2004) and Lindgren et al. (2007). This may represent differences between laboratory and field conditions or differences in developmental stages that are known to affect allocations to these defensive chemicals (Bråthen et al. 2004).

Total phenolics responded more as expected, with two species (a grass and a forb) showing increased TP in response to simulated herbivory. At least one other grass, western wheatgrass (*Pascopyrum smithii*), has shown an

Table 2 Means and standard errors (in parentheses) for chemical assays of forage plants at different levels of simulated herbivory

	Control	Low	High	Sustained
Concentration of proteinase inhibitor (mg/gdm)				
C. bigelowii	9.3 (0.97)a	9.0 (1.06)a	2.6 (0.51)b	3.3 (0.47)b
A. odoratum	3.7 (0.39)	3.5 (0.42)	4.7 (0.37)	3.8 (0.40)
D. cespitosa	3.5 (0.49)	3.7 (0.42)	4.5 (0.19)	3.8 (0.20)
D. flexuosa	2.8 (0.43)	2.7 (0.39)	3.1 (0.48)	2.4 (0.37)
G. sylvaticum	2.8 (0.44)	3.9 (0.34)	3.6 (0.37)	3.1 (0.34)
R. acetosa	4.6 (0.33)	4.6 (0.60)	3.5 (0.45)	3.7 (0.20)
S. virgaurea	3.9 (0.36)a	6.8 (0.62)b	4.6 (0.41)a	3.3 (0.25)a
V. myrtillus	4.0 (0.40)	5.2 (0.42)	4.6 (0.31)	4.0 (0.48)
Concentration of total soluble protein (mg/gdm)				
C. bigelowii	22.0 (4.35)	19.3 (2.81)	17.3 (2.52)	19.7 (2.84)
A. odoratum	17.1 (4.41)	19.6 (2.38)	20.6 (5.32)	17.8 (3.62)
D. cespitosa	19.6 (3.74)	19.3 (4.21)	16.1 (2.25)	17.3 (2.60)
D. flexuosa	22.0 (2.27)a	22.2 (1.30)a	22.6 (1.59)a	12.6 (2.78)b
G. sylvaticum	17.0 (6.25)	13.3 (7.18)	15.3 (5.91)	17.6 (5.09)
R. acetosa	6.0 (1.10)a	8.4 (1.93)ab	11.5 (3.26)b	7.1 (3.14)a
S. virgaurea	14.4 (3.32)	13.3 (2.78)	12.6 (3.15)	9.8 (3.72)
V. myrtillus	18.1 (5.39)ab	15.8 (1.42)ab	19.0 (4.68)a	14.9 (3.70)b
Ratio of proteinase inhibitor to soluble plant proteins				
C. bigelowii	0.35 (0.02)a	0.49 (0.08)a	0.12 (0.03)b	0.17 (0.03)b
A. odoratum	0.22 (0.02)	0.18 (0.02)	0.25 (0.04)	0.22 (0.03)
D. cespitosa	0.18 (0.02)a	0.20 (0.02)a	0.29 (0.03)b	0.22 (0.01)ab
D. flexuosa	0.13 (0.02)	0.12 (0.02)	0.14 (0.02)	0.20 (0.04)
G. sylvaticum	0.17 (0.02)	0.28 (0.05)	0.26 (0.04)	0.19 (0.04)
R. acetosa	0.79 (0.05)a	0.57 (0.08)ab	0.34 (0.07)b	0.59 (0.07)ab
S. virgaurea	0.28 (0.04)a	0.53 (0.07)b	0.38 (0.05)ab	0.39 (0.07)ab
V. myrtillus	0.22 (0.03)a	0.33 (0.04)b	0.26 (0.04)a	0.27 (0.02)ab
Concentration of total phenolics (mg/gdm)				
C. bigelowii	7.1 (0.90)ab	6.3 (0.54)b	9.6 (0.79)a	5.4 (0.65)b
A. odoratum	1.9 (0.17)a	2.9 (0.23)b	2.6 (0.10)b	2.4 (0.16)ab
D. cespitosa	2.1 (0.20)	2.2 (0.06)	1.9 (0.15)	2.5 (0.25)
D. flexuosa	4.4 (0.50)	3.9 (0.23)	3.8 (0.14)	4.6 (0.28)
G. sylvaticum	19.7 (0.82)ab	20.8 (0.48)a	17.3 (0.58)b	24.8 (0.76)c
R. acetosa	9.6 (0.72)a	6.5 (0.80)b	6.3 (0.83)b	5.7 (0.77)b
S. virgaurea	2.3 (0.74)	2.1 (0.54)	1.6 (0.21)	1.5 (0.30)
V. myrtillus	26.5 (1.08)a	26.4 (0.55)a	26.5 (0.80)a	23.2 (0.52)b
Ratio of total phenolics to soluble plant proteins				
C. bigelowii	0.36 (0.07)ab	0.33 (0.03)ab	0.57 (0.06)a	0.28 (0.04)b
A. odoratum	0.11 (0.01)	0.15 (0.01)	0.14 (0.02)	0.14 (0.02)
D. cespitosa	0.11 (0.01)	0.12 (0.01)	0.12 (0.01)	0.15 (0.02)
D. flexuosa	0.20 (0.02)a	0.17 (0.01)a	0.17 (0.01)a	0.38 (0.04)b
G. sylvaticum	0.80 (0.21)	2.10 (0.54)	1.36 (0.29)	1.57 (0.12)
R. acetosa	1.69 (0.27)a	0.82 (0.14)b	0.56 (0.05)b	0.84 (0.08)b
S. virgaurea	0.15 (0.03)	0.18 (0.06)	0.13 (0.02)	0.17 (0.04)
V. myrtillus	1.61 (0.25)	1.68 (0.08)	1.28 (0.04)	1.66 (0.19)

N=6 for all means. Different letters indicate significant differences between treatments for that species (P<0.05)

induced response in TP, in this case in response to heavy defoliation by grasshoppers (Redak and Capinera 1994). Still, most of our species did not exhibit the expected result. The reduction in TP in *Vaccinium* in response to sustained clipping has also been reported previously for *Vaccinium* on islands in northern Norway where repeated damage from voles led to reduced levels of TP (Oksanen et al. 1987).

Some of the results from our study could reflect the use of artificial clipping. Mechanical wounding may not stimulate the plant in the same way as natural herbivory (Baldwin 1990). Several studies have found plants to respond differently to mechanical wounding either in amount of response (Haukioja and Neuvonen 1985; Faeth 1986; Baldwin 1988) or in kind of response (Hartley and Lawton 1987; Neuvonen et al. 1987; Pontoppidan et al. 2005) compared to natural herbivory. Some plants may respond not only to the loss in leaf tissue, but also to some component of the herbivore saliva (Coleman et al. 2007) or pathogen invasion (Kavitha and Umesha 2008) at the site of wounding that is not replicated in simulated herbivory trials. However, the use of mechanical wounding does replicate results from natural herbivory in some systems. The response of *Vaccinium* in our study clearly conformed to the pattern seen in natural systems, and others have found simulated herbivory to provide a good indication of the effects of natural herbivory (Green and Ryan 1972; Karban 1986; Neuvonen et al. 1987). The applicability of simulated herbivory for predicting responses to natural herbivory likely depends on the species in question and the type of herbivory being investigated. Because it is easier to control, simulated herbivory, thus, provides a good starting point for further investigations in the field.

Variation among species in this study was larger than that seen for any treatment effects within a species. The level of secondary metabolites was relatively high for several of the species, all of which were preferred forage species for both sheep and voles. For instance, the level of PI in all species except *D. flexuosa* was higher than that avoided by lemmings in Canada (Bråthen et al. 2004). The levels of TP in both *Geranium* and *Vaccinium* were relatively high, yet voles (Saetnan et al. 2009) and sheep (Mysterud and Austrheim 2005) readily consumed both species. If palatable plants naturally have such high levels of secondary metabolites, it is unlikely that smaller effects in response to damage by herbivores would have a significant effect on diet choice.

Of course, we only examined two types of defensive compounds, and other chemicals could be important as induced defenses against mammalian herbivory. Recent studies on grasses have shown that increased accumulation of silica in response to grazing can affect the growth and reproduction of voles, which could influence their population dynamics (Massey and Hartley 2006; Massey et al. 2007). This possibility deserves further investigation, but our results do not support the hypothesis that herbivory induces a long-term increase of defensive compounds in important forage plants on alpine rangelands of Norway, a result consistent with those from field studies.

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RAPID COMMUNICATION

Changes in Nutritional Value of Cyanogenic *Trifolium repens* Grown at Elevated Atmospheric CO_2

Roslyn M. Gleadow · Everard J. Edwards · John R. Evans

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Abstract Global food security in a changing climate depends on both the nutritive value of staple crops as well as their yields. Here, we examined the direct effect of atmospheric carbon dioxide on the toxicity of the important pasture crop, *Trifolium repens* L. (clover). Shoots of *T. repens* contain cyanogenic glycosides that break down to release toxic hydrogen cyanide when damaged. The ability of animals to tolerate cyanogenic compounds is dependent, in part, on their overall protein intake. We grew *T. repens* communities at ambient and approximately twice-ambient CO_2 in a controlled environment greenhouse experiment. We found that the ratio of total cyanogenic glycosides to total protein ratio was nearly two times higher in leaves of *T. repens* grown at elevated CO_2. This study highlights the importance of assessing the nutritive value of this and other plants in response to rising CO_2 so that steps can be taken to address any adverse consequences for herbivores.

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Introduction

Two major problems facing the world today are climate change and population growth. Despite the large body of research on the effect of elevated CO_2 on primary productivity, few studies consider the overall nutritional value of plants. Typically, leaf nitrogen and protein concentrations are 10–20% lower in plants grown at twice-ambient CO_2 (Ainsworth and Long 2005; Taub et al. 2008). In addition, concentrations of carbon-based defense compounds such as phenolics are often higher (Lincoln et al. 1993), again decreasing the nutritive value.

Less is known about the effect of elevated CO_2 on cyanogenic glycosides. Cyanogenic glycosides are constitutive defense compounds found in about 5% of all plants (Jones 1998; Gleadow and Woodrow 2002). When plant tissue containing cyanogenic glycosides is crushed or chewed, the glycosides are mixed with endogenous β-glucosidases, and toxic hydrogen cyanide (HCN) is released. Given the high proportion of crop plants that are cyanogenic (c. 60%, Jones 1998), it is vital to know whether or not such plants will become more cyanogenic in the future and to compare that with predicted decreases in leaf protein levels.

Trifolium repens (white clover) is an important component of pastures in many parts of the world. It contains two cyanogenic glycosides, mostly linamarin (α-hydroxyisobutyronitrile-β-D-glucopyranoside) and a smaller proportion of lotaustralin (2-hydroxy-2-methylbutyronitrile-beta-D-glucopyranoside). To our knowledge, only one elevated CO_2 study of *T. repens* has included an analysis of the

endogenous cyanogenic glycosides (Frehner et al. 1997). Those results were inconclusive, as there was a lot of seasonal variation, and the workers did not measure leaf nitrogen. Gleadow et al. (1998), in a study of cyanogenic *Eucalyptus cladocalyx*, found that cyanogenic glycoside concentration per mass did not increase in leaves of seedlings grown at elevated CO_2, but there was a 40% decrease in leaf protein, increasing the overall leaf toxicity. The aim of this study, therefore, was to quantify the protein and cyanogenic glycoside concentrations in *T. repens* grown under ambient and twice-ambient CO_2 in order to predict the ability of pastures to support grazing animals in the future.

Methods and Materials

This experiment was part of a larger study described in detail by Edwards et al. (2006). Briefly, *Trifolium repens* cv. Haifa L. was grown in 36 plastic containers (525×370×450 mm) filled with steam-treated sand in temperature-controlled, naturally lit greenhouses (25°C/20°C; day/night) for 15 months. Each container was inoculated with *Rhizobium leguminosarum* bv. Trifolii strain ANU843. Greenhouses were supplied with air containing ambient (360 ppm) or twice-ambient (700 ppm) CO_2. Pots were watered daily and fertilized three times per week with 500 ml Rorison's solution, with the N components adjusted to contain low or high NO_3 (0 or 4 mM nitrate) (N=6). Cyanogenic glycosides were measured as evolved HCN on subsamples of freeze-dried leaf material, expressed as mg CN g^{-1}_{DW} (Woodrow et al. 2002). Remaining leaves were oven dried and then analyzed for total leaf nitrogen by using an EA 1110 CHN-O Carlo-Erba Elemental Analyzer (Carbo-Erba instruments, Milan, Italy) and total protein with the Lowry method. Data were analyzed with Minitab15.

Results and Discussion

This is the first study on the impact of elevated CO_2 on nutritional quality in cyanogenic pasture plants. We found that leaf nitrogen was c. 20% lower in foliage of *T. repens* grown at elevated CO_2 (P<0.05; Fig. 1a), and protein concentration was c. 25% lower (P<0.01; Fig. 1b). These findings are consistent with other studies of many other C3 plants, and usually those reflect some degree of down-regulation in photosynthesis (Ainsworth and Long 2005). Such acclimation is usually more pronounced when growth rates are limited by nitrogen supply (Gleadow et al. 1998; Ainsworth and Long 2005). In our study, there was no significant difference in the response to elevated CO_2 between the two N treatments, probably because *T. repens*

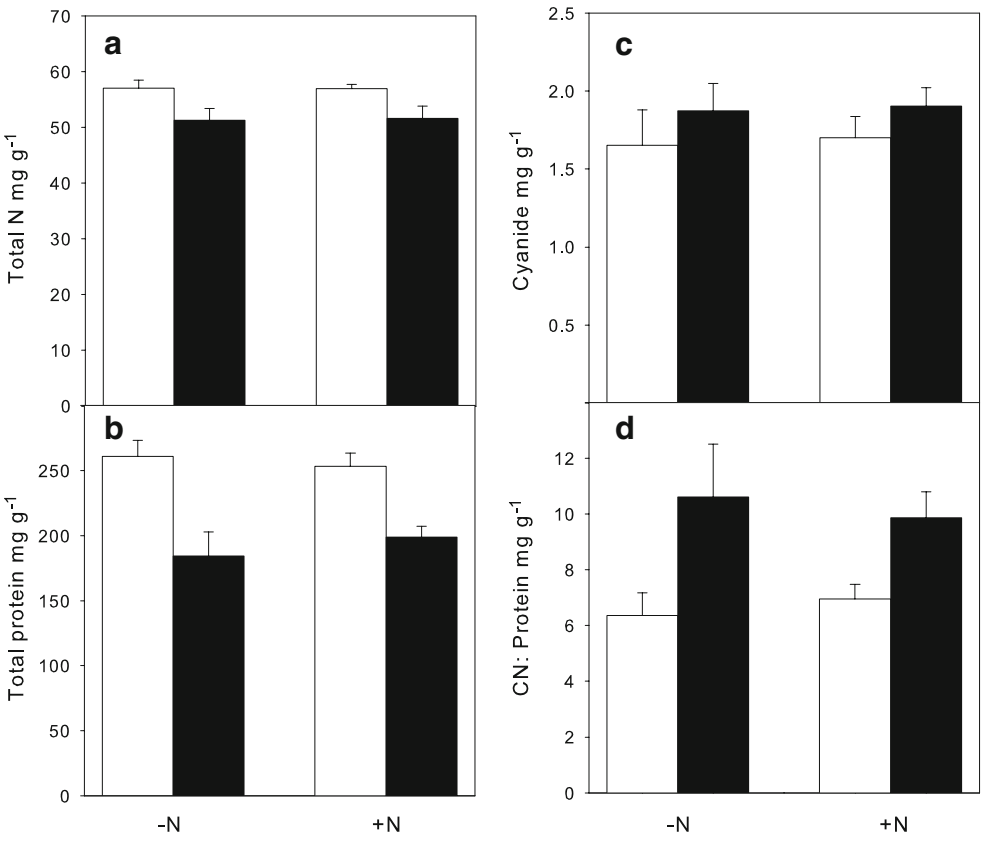

Fig. 1 Chemical composition of *Trifolium repens* grown at ambient (*open*) and elevated CO_2 (*closed*) and supplied with nutrient solutions containing nitrate (+N) or without nitrate (-N). **a** Total leaf nitrogen; **b** total protein; **c** cyanogenic glycosides (measured as evolved cyanide); **d** ratio of evolved cyanide to total leaf protein. All data are expressed on a dry weight basis

supplements its N requirements by fixing atmospheric N through symbiotic associations with *Rhizobium* bacterium (Edwards et al. 2006).

There have been, to our knowledge, only three published studies on the effect of atmospheric CO_2 on cyanogenic glycosides. We found here that total cyanogenic glycoside concentration in *T. repens* was not significantly different in plants grown at ambient and twice-ambient CO_2 (Fig. 1c), consistent with these earlier studies (Frehner et al. 1997; Gleadow et al. 1998; Bazin et al. 2002). By contrast, we did not detect a significant impact of the nitrate supply on cyanogenic glycoside concentration as has been observed by others (Gleadow et al. 1998), reflecting the consistent leaf N concentrations across treatments.

Animals have the ability to convert cyanide to the less toxic thiocyanate, but the capacity to do this is dependent on the rate of ingestion and the availability of sulfur-rich proteins (Westley 1988; Gleadow and Woodrow 2002). We calculated the cyanide-to-protein ratio as an index of the nutritional quality of *T. repens* grown at elevated CO_2. The amount of cyanide relative to protein increased by 40% in plants supplied with NO_3 and by 30% in plants not given additional NO_3 (Fig. 1d; $P<0.001$), although the NO_3 effect alone was not statistically significant. Forage with greater than 0.8 mg CN g^{-1}_{DW} generally is considered to be toxic to cattle under field conditions (Westley 1988), about half the value reported here (overall mean=1.7 mg CN g^{-1}_{DW}). Typically, *T. repens* occurs in mixed pastures, and animals would be unlikely to ingest toxic quantities. However, if cyanogenic glycoside concentration increases relative to the protein in the future, then the overall nutritional value of the pasture would be substantially less.

Protein content of food crops such as wheat and rice are predicted to contain to 15–20% less protein by the end of this century (Taub et al. 2008). Lower protein content of leaves of pasture plants is also of concern as this would mean that grazing animals would need to increase their consumption in order to maintain their current protein intake. Insect studies have shown that animals compensate for the lower protein content of plants grown at elevated CO_2 by eating more (Lincoln et al. 1993). If this is also true of grazing mammals, then they would ingest more cyanogenic glycosides along with the rest of the plants in mixed pastures. Other species in the pasture are likely to have higher concentrations of antifeedants such as phenolics as well, which are known to interfere with protein uptake (Lincoln et al. 1993; Taub et al. 2008). If these results hold true for other cultivars, it is possible that pastures rich in *T. repens* could become unsuitable for livestock if atmospheric CO_2 continues to increase. These results demonstrate the importance of testing the effect of climate change on the nutritive value of crop plants as well as yield.

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Pretreatment of Clover Seeds with Nod Factors Improves Growth and Nodulation of *Trifolium pratense*

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Abstract The increase in legume production in sustainable agriculture depends not only on the effectiveness of the selected nitrogen-fixing inoculants but also on their competitiveness in a soil environment containing an indigenous rhizobial population. In this study, we investigated the effect of pretreatment of red clover seeds with specific Nod factor (LCOs) on germination, growth, and nodulation of clover growing under sterile conditions and in the soil. We demonstrated that, although the symbiotic ability and competitiveness of the inoculant strain RtKO17 was not improved under competitive soil conditions, LCOs treatment of clover seeds significantly enhanced clover nodulation and growth of plants.

Keywords Competitiveness · Nod factor · Symbiosis · *Rhizobium leguminosarum* bv. *trifolii*

Introduction

Rhizobia are gram-negative soil bacteria able to establish symbiosis with leguminous plants and reduce atmospheric nitrogen to ammonia that in turn is used by plants. Nodule formation is a highly specialized process that requires "cross talk" between the bacteria and the host plant. The first step of the interaction includes release of the host plant signal molecules, usually specific flavonoids, and the synthesis of bacterial signal molecules, lipochitooligosaccharides (LCOs), called Nod factors (for review, see Spaink 2000; D'Haeze and Holsters 2002). The Nod factors consist of a chitooligosaccharide backbone of three to five β-1,4-linked *N*-acetyl-D-glucosamine residues, substituted by a fatty acyl chain of varying lengths and with varying degrees of unsaturation, attached at the nonreducing end. Other diverse substitutions controlled by specific *nod* genes and present on both ends of the oligochitin chain make the Nod factors specific for plant hosts (Denarie and Cullimore 1993; Spaink et al. 1994). The biosynthesis of Nod factors is controlled by *nod* genes transcriptionally induced by a flavonoid-activated NodD protein. Recognition by the NodD protein of the particular flavonoids determines the first level of specificity in the symbiosis. Nod factors are secreted and elicit multiple responses in the root epidermis, cortex, and pericycle that lead to the nodulation of the appropriate host plants (Heidstra and Bisseling 1996). One of the earliest plant responses to Nod factors is an increase in the intracellular calcium level in root hairs, followed by calcium oscillations and alteration of the root hair cytoskeleton (for review, see Heidstra and Bisseling 1996; Gage 2004; Jones et al. 2007). These responses are followed by curling of root hairs to trap rhizobia and stimulation of root cortex cells to reinitiate mitosis. The rhizobia invade the roots through tubular structures called infection threads, which penetrate the root cortex and stimulate the nodule primordium formation. The nodule primordium develops into a nodule, while the bacteria differentiate into their endosymbiotic form, the bacteroids, that are able to fix nitrogen into ammonia, which are subsequently utilized by the plant.

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Structurally appropriate Nod factors, apart from exopolysaccharide production, are required for an ongoing infection thread formation and elongation, as well as other plant responses in the indeterminate nodule-forming plants (Jones et al. 2007). Application of purified Nod factors to legume roots at nanomolar concentrations can elicit root hair deformation, cortical cell divisions, and in some cases, the formation of nodule-like outgrowth (Heidstra and Bisseling 1996).

The Nod factors of *Rhizobium leguminosarum* bvs. *trifolii* and *viciae* consist of three to five residues of a *N*-acetyl-D-glucosamine backbone to which a fatty acid chain is attached at the nonreducing end and various substituents that occur on the chitin-like backbone (Spaink et al. 1991). *R. leguminosarum* bv. *trifolii* produces a subset of Nod factors that bear a complex mixture of highly unsaturated fatty acyl chains, including $C_{18:3}$, $C_{20:3}$, and $C_{20:4}$, determined by the protein products of the region downstream of *nodEF* and *nodRL* (Bloemberg et al. 1995; Schlaman et al. 2006). The overall hydrophobicity of the highly unsaturated or hydroxylated fatty acyl moieties of the Nod factors has been shown to be essential for host-specific biological activity. Nod factors are structurally diverse and a single rhizobial strain may produce a range of metabolites (Spaink et al. 1995). They are recognized by plant receptors (Oldroyd and Downie 2004) and have a mitogenic effect, inducing the cortical cell division potential, thus leading to the formation of nodule primordium (Spaink et al. 1991). Purified LCOs can reinitiate cell division, giving rise to nodule-like structures (Stokkermans et al. 1995). There is also evidence that Nod factor (Nod Bj V C18:1, MeFuc) produced by *Bradyrhizobium japonicum*, as well as a number of synthetic LCOs, improve germination and early growth of soybean and nonlegume plants tested in greenhouse and in short-term field experiments (Prithiviraj et al. 2003).

Attempts to improve nodulation and nitrogen fixation in the soil often fail because of the out competition of inoculant strains by the indigenous rhizobial population that can generally be less efficient in nitrogen fixation but better adapted, with a higher infectivity potential, resulting in higher nodule occupancy (for review, see Triplett and Sadowsky 1992; Streeter 1994; Toro 1996). Thus, rhizobial strains used as inocula must not only be effective in nitrogen fixation but also highly competitive. In our previous work, we selected from among the indigenous soil rhizobial population *R. leguminosarum* bv. *trifolii* strains that were competitive and highly responsive to flavonoid induction (Wielbo et al. 2007, Maj et al. 2008).

The main objective of the current study was to assess whether a pretreatment of clover seeds with specific LCOs improves germination, growth, and nodulation under sterile conditions and whether this treatment affects the symbiotic ability and competitiveness of the inoculant strain under competitive soil conditions. We hypothesized that clover germination and growth improvement by LCO treatment, even under the competitive conditions, could facilitate a subsequent inoculation with the rhizobial strain RtKO17 previously selected as competitive and effective in nodulation and nitrogen fixation. Under these conditions, plant roots exposed to LCOs prior to infection would have been infected more readily by the inoculant strain than by its less robust competitors. To our knowledge, the effect of LCO treatment on clover growth and nodulation has not been studied thus far.

Methods and Materials

Bacterial Strains and Growth Conditions

R. leguminosarum bv. *trifolii* strain KO17 (RtKO17) was isolated from nodules of red clover (*Trifolium pratense* L. cv. Dajana) grown on cultivated soil in the region of Lublin, Poland. In the competition experiment, the derivative of RtKO17 carrying pJBA21Tc with constitutively expressed *gusA* gene was used (Wielbo et al. 2007). Rhizobial strains were maintained and grown on mannitol yeast extract agar 79CA medium (Vincent 1970). *Escherichia coli* strains were grown on Luria–Bertani (LB) medium (Sambrook et al. 1989). When needed, tetracycline was added to 79CA or LB media at the concentration 10 μg/mL.

Preparation of Exudate from Sprouted Seeds

Red clover (*T. pratense* L. cv. Dajana) seeds were surface-sterilized by immersion in 0.1% $HgCl_2$ for 3 min, rinsed with sterile distilled water, then treated with 70% ethanol for 3 min, followed by extensive washing with sterile water. The sterilized seeds were shaken in sterile water, in darkness, for 4 days at 28°C, and during this time, the seeds germinated. After the removal of the sprouted seeds, the supernatant was extracted with ethyl acetate in the ratio 10:1 (*v*/*v*). Ethyl acetate was evaporated, and the pellet was resolubilized in 95% ethanol and stored at 4°C. To determine the amount of the flavonoids, the ethanol extracts were dried and weighed. The approximate flavonoid concentration in seed exudate was calculated relative to the molecular weight of authentic flavone.

LCOs Isolation

Logarithmic cultures of *R. leguminosarum* bv. *trifolii* strain KO17 were induced with sterile clover seed exudate at a final concentration of 2 μM and incubated at 28°C for an additional 48 h. One liter of the activated culture was

extracted twice with 0.2 volume of *n*-butanol (Prithiviraj et al. 2003). The organic fraction was separated and dried under vacuum in a rotary evaporator (Rotavapor–R, Bűchi, Switzerland). The amount of Nod factors was determined by conversion of the amino sugars to methyl glycosides and gas chromatography/mass spectrometry (GC/MS) analysis. To 250 µg sample, 30 µg GalNAc was added as internal standard and hydrolyzed in 1 mL of 2 M trifluoroacetic acid (TFA) at 120°C for 2 h. The TFA was eliminated by washing the samples two times with Millipore water, following which the samples were dried under nitrogen. Free fatty acids were removed from the samples with 0.5 mL 10% (*v*/*v*) ether in hexane. This step was repeated three times. Samples were reduced by solid sodium borohydride ($NaBH_4$) at 0°C in darkness for 12 h. The excess of $NaBH_4$ was removed by sequential distillation with the following solutions: 200 µL 10% (*v*/*v*) acetic acid in water, 200 µL 10% (*v*/*v*) acetic acid in methanol, and 200 µL 1 M HCl in methanol. Samples were redistilled two times with methanol and dried under nitrogen. In the next acetylating step, 25 µL acetic anhydride and 50 µL pyridine were added to the dry sample and incubated at 85°C for 30 min and then dried under nitrogen. The sample was dissolved in a small volume of chloroform and applied to GC/MS (Hewlett-Packard HP 5890A) equipped with an HP.5MS capillary column coupled to a mass selective detector MSD HP 5971. The temperature program was as follows: initially 150°C for 5 min, then raised to 310°C (5°C/min), and the final temperature 310°C for 10 min. LCOs concentration was approximated based on the assumption that a single molecule of a Nod factor contains on average four residues of GlcNAc. The calculated content of GlcNAc in the LCOs preparation was 346 nM.

Root Hair Deformation Assay

To estimate the biological activity of isolated LCOs, a root hair deformation test was performed on red clover (*T. pratense* L. cv. Dajana). Surface-sterilized seeds of clover were germinated on Fåhraeus agar medium (Fåhraeus 1957) in Petri dishes in the dark at 28°C. After 24 h incubation, seedlings were transferred to microscope glass slides (two seedlings per slide) covered with 1 mL 0.4% Fåhraeus agar medium supplemented with serially diluted *R. leguminosarum* bv. *trifolii* LCO at the final concentration from 10^{-8} to 10^{-13} M. The initial content of GlcNAc in the LCOs preparation was 346 nM. Slides were placed in a moist chamber and incubated at 28°C in the dark. After 24 and 48 h, the growth and deformation of individual root hairs was assessed under a light microscope (Olympus BX41). Root hairs of at least ten plants were examined for each LCO dilution.

Radioactive Labeling of LCOs and TLC

To label LCOs in vivo, *R. leguminosarum* bv. *trifolii* strain KO17 was grown in M1 medium containing 18.7 mM NH_4Cl, 11.5 mM K_2HPO_4, 3.7 mM KH_2PO_4, 1.7 mM NaCl, 0.8 mM $MgSO_4 \cdot H_2O$, 0.01 mM $FeCl_3$, 1% glycerol, 1 µg/mL thiamine HCl, 0.5 µg/mL biotin, and 1 µg/mL pantothenate at 28°C to OD_{550} 0.4–0.5. One milliliter cultures were supplemented with clover seed exudate at a final concentration of 2 µM and incubated at 28°C for 2 h; 12.5 µCi [2-C^{14}] sodium acetate (50–60 mCi/mmol; Hartmann Analytic, Braunschweig, Germany) were added and further incubated for 16 h at 28°C. Radioactive LCOs were extracted with *n*-butanol and concentrated by evaporation. Pellets were dissolved in 40 µL *n*-butanol, and volumes of 1 µL were analyzed on reversed-phase C18-coated TLC silica plates (Merck, Darmstadt, Germany) using acetonitrile–water (1:1, *v*/*v*) as the mobile phase (Spaink et al. 1992). TLC plates were dried and exposed to Kodak X-OMAT K film for 45 days at room temperature.

Clover Germination in the Presence of LCOs

Seeds of clover (*T. pratense* L. cv. Dajana) were surface-sterilized and soaked in serially diluted LCO solution (from 10^{-7} to 10^{-13} M) for 30 min. Seeds were transferred to Petri dishes with Fåhraeus agar medium (50 seeds per dish) and placed in darkness at 28°C. Each treatment was conducted in three replicates. Germination was observed after 24 and 48 h, and the root length and the percentage of germinated clover seedlings were estimated.

Clover Growth Assays in the Presence of LCOs

Sterile seeds of clover were soaked in serially diluted LCOs as described above and transferred to plastic pots with sterile sand (600 g of sand per pot, ten seeds per pot). Pots were irrigated once with 50 mL Fåhraeus medium supplemented with 5 mM KNO_3 and then watered every 2 days. The growth chamber was set at a 16/8-h light/dark (L/D) regime and at 22°C/15°C day/night temperature. After 6 weeks of growth, clover plants were harvested and fresh and dry mass of shoots were estimated. Each treatment was carried out in ten replicates, and the experiment was repeated twice with similar results.

Effect of LCOs on *T. pratense–R. leguminosarum* bv. *trifolii* Symbiosis: The Greenhouse Experiment

Four different treatments of sterilized seeds of clover (*T. pratense* L. cv. Dajana) were prepared: (1) seeds soaked in water for 30 min; (2) seeds soaked in LCO solution (~10^{-10} M) for 30 min; (3) seeds soaked in water

and inoculated with 10 mL suspension of RtKO17 (~7×10^7 CFU/mL); (4) seeds soaked in LCO solution and inoculated with 10 mL suspension of RtKO17. Seeds were sown in plastic pots with sterile sand (600 g of sand per pot, ten seeds per pot). Pots were watered every 2 days. The growth chamber was set at a 16/8-h L/D regime and at 22°C/15°C day/night temperature. After 6 weeks, the plants were harvested, and the fresh and dry mass was estimated. Each treatment was carried out in five replicates, and the experiment was repeated twice with similar results.

Outdoor Experiment

The experiment was conducted at the Institute of Soil Science and Plant Cultivation, Puławy, Poland. Plants were cultivated in Mitcherlich pots filled with 7 kg of loamy sand (mixture of sand and loamy soil in the ratio 2:1). Pots were kept under a rainout shelter for 6 weeks in May and June 2008. The soil was maintained at about 60% water-holding capacity. In this experiment, the strain RtKO17 (pJBA21Tc) tagged with *gusA* marker was used to study its competitiveness in relation to indigenous rhizobia.

Four different treatments of nonsterilized seeds of clover (*T. pratense* L. cv. Dajana) were conducted: (1) seeds soaked in water for 30 min and sown in pots (20 seeds of clover per pot) to test clover infection by autochthonous rhizobia; (2) seeds soaked in LCO solution (~10^{-10} M) to test clover infection by autochthones in the presence of LCO; (3) seeds inoculated with 10 mL suspension of RtKO17(pJBA21Tc) (~7×10^7 CFU/mL) to test the competitiveness of the inoculant; (4) seeds soaked in LCO solution (10^{-10} M) and inoculated with 10 mL RtKO17 (pJBA21Tc) (~7×10^7 CFU/mL) to test for the effect of LCOs on inoculant competitiveness. Each treatment had seven replicates. After 2 weeks, clover from one pot per treatment was inspected for number of nodules, and the roots were stained for GUS^+ activity (in groups 3 and 4). Overall, 24 pots were examined, with 20 clovers per pot. After 6 weeks of growth, plants were removed, and the roots were gently washed in tap water, the shoots and roots were separated, and their fresh and dry mass was estimated. Samples of roots were stained for β-glucuronidase (GUS) activity in 50 mmol/L^{-1} sodium phosphate buffer (pH 7.2) with 50 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Glc), 0.1 mmol/L ethylenediaminetetraacetic acid, 0.38 mmol/L $K_3Fe(CN)_6$, and 0.38 mmol/L $K_4Fe(CN)_6$ for approximately 12 h at room temperature (Wilson et al. 1995). The competitive potential of strain RtKO17(pJBA21Tc) was determined by calculating the number of blue nodules (colonized by the *gusA*-tagged strain) and the number of white nodules (elicited by indigenous rhizobia).

Statistical Analysis

Statistical analysis was carried out by the analysis of variance and Tukey test using the STATISTICA software (Stat Soft, Tulsa, OK, USA).

Results

Biological Activity of LCOs Produced by Strain RtKO17

In this study, we used the *R. leguminosarum* bv. *trifolii* strain KO17 (RtKO17) which was previously selected as effective in plant growth promotion and a competitive field isolate (Wielbo et al. 2007; Maj et al. 2008). To isolate Nod factors (LCOs), strain RtKO17 growing in M1 medium supplemented with radioactive C^{14}-acetate was induced with red clover seed exudate instead of synthetic flavonoids in order to obtain less artificial conditions. LCOs were isolated as *n*-butanol extracts, and the samples were run on TLC plates for estimation of LCOs quality. Under the described conditions, after the induction of RtKO17 for 48 h, at least five spots were visible in comparison to the noninduced control (Fig. 1), signifying that different types of LCOs were produced. Comparison of LCOs production profile of flavonoid-induced RtKO17 with the previously published *R. leguminosarum* LCOs profiles (Tak et al. 2004) indicates that the spots could represent several forms of LCOs, likely consisting of four to five glucosamine

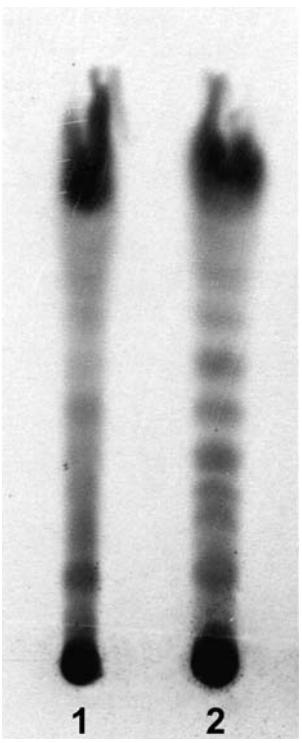

Fig. 1 Autoradiogram of thin-layer chromatography separation of LCOs from *R. leguminosarum* strain KO17 labeled with [2-C^{14}] sodium acetate. Cells were grown without inducer (*1*) or in the presence of 2 μM of flavonoid inducer for 48 h at 28°C (*2*)

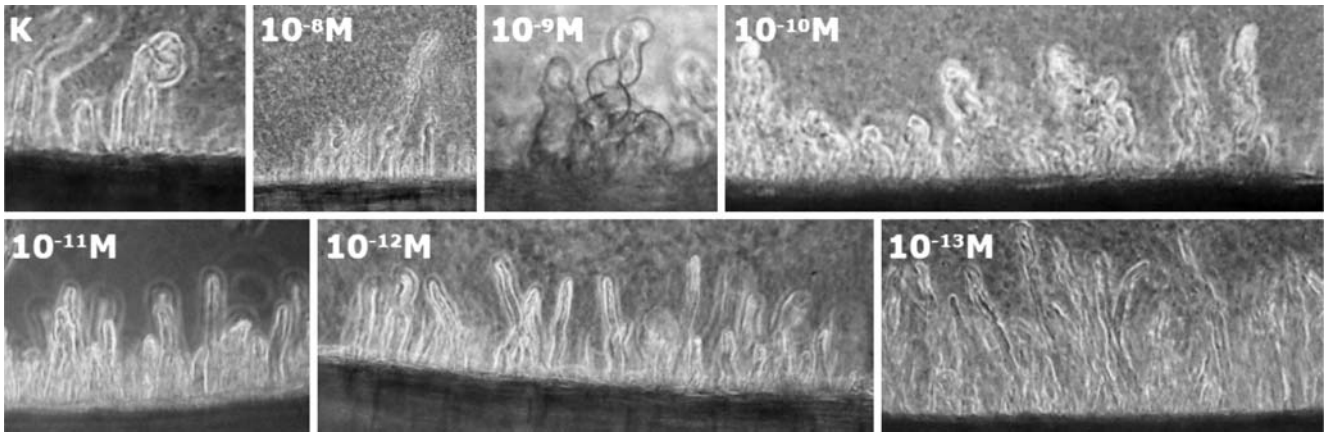

Fig. 2 Root hair deformation induced by appropriate concentration of LCOs isolated from *R. leguminosarum* strain KO17. Control showing typical root hair deformation after addition of strain RtKO17

residues bearing a complex mixture of highly unsaturated fatty acyl chains. For plant test assays, nonlabeled LCOs were isolated from a large volume of a culture growing under comparable conditions. The approximate amount of LCOs was estimated by GC chromatography, and its biological activity was examined in a root hair deformation assay (Heidstra et al. 1994). Crude *n*-butanol extract of RtKO17 flavonoid-induced culture was serially diluted, and clover root hair deformation was examined by microscopic observations as described in the "Methods and Materials" section. Root hair curling could be observed over a range of LCOs concentration (Fig. 2), but the most pronounced deformation occurred within the range 10^{-9} to 10^{-10} M.

Using LCOs isolated from RtKO17, we examined the effect of different concentrations of Nod factor on the germination of clover on Fåhraeus agar plates (Table 1). After seed treatments for 24 and 48 h, the germination and root lengths increased significantly when compared to the untreated control. The strongest and statistically significant effect both on germination and root length of clover was observed with the range 10^{-10} to 10^{-12} M LCOs after 2 days incubation.

Next, we examined the effect of LCOs treatment on the germination and growth of surface-sterilized clover seeds grown for 6 weeks in sterile sand under laboratory conditions. A statistically significant stimulation of shoot growth was found with 10^{-9} to 10^{-11} M LCOs and of roots with 10^{-9} to 10^{-12} M LCOs, whereas the differences in seed germination were not statistically significant (Table 2).

The experiments described above allowed us to determine the concentration of LCOs produced by strain RtKO17 that substantially stimulated the germination and growth of clover seedlings under sterile laboratory conditions during long-time cultivation. The most active concentration of crude extract of LCOs produced by strain RtKO17 was estimated at approximately 10^{-10} M.

Effect of LCOs on Growth and Nodulation of Clover Grown in Sterile Sand Inoculated with Strain RtKO17: The Greenhouse Experiment

We examined the growth and nodulation of clover seeds treated with LCOs (~10^{-10} M) grown in sterile sand and inoculated with the strain RtKO17 in comparison to uninoculated control plants (Table 3). In the case of LCO-treated seeds that were not inoculated with rhizobia (groups 1 and 2), a moderate stimulation of shoot growth was observed. Under these conditions, LCO treatment stimulated the growth of clover, however, the fresh and dry shoot mass was not statistically different when compared with seeds soaked in water and inoculated with the same strain (treatment groups 3 and 4). The number of nodules was also not significantly different in relation to control plants (Table 3).

Table 1 Effect of *R. leguminosarum* bv. *trifolii* KO17 Nod factor (LCOs) on the germination of clover seeds (*T. pratense*)

LCOs concentration (M)	Germinated seeds (%)	Length of roots (mm)	Germinated seeds (%)	Length of roots (mm)
	24h		48h	
Control	58±14a	3.1±0.5a	63±12a	11.8±4.6a
10^{-8}	54±11a	3.3±1.1a	59±13a	13.6±4.1a
10^{-9}	59±1a	3.2±0.5a	85±5b	15.5±3.0ab
10^{-10}	83±2b	3.9±0.6a	92±2b	17.5±3.4b
10^{-11}	85±4b	4.8±0.3b	89±3b	18.6±2.7b
10^{-12}	88±8b	3.6±0.4a	96±4b	15.8±2.7ab
10^{-13}	67±3a	3.4±0.4a	85±7b	14.9±4.2ab

Values are the mean±standard deviation of 150 clover seeds in each treatment. Means within the same column and followed by the same letter are not significantly different ($P>0.05$)

Table 2 Effect of *R. leguminosarum* bv. *trifolii* KO17 LCOs on the growth of clover (*T. pratense*) grown in sterile sand under greenhouse condition

LCOs concentration (M)	Germination (days)	Fresh mass of shoots (mg)	Fresh mass of roots (mg)
Control	5.0±0.4a	47.8±5.1a	21.5±2.9a
10^{-8}	5.0±0.3a	57.3±5.9a	37.3±4.7b
10^{-9}	5.4±0.6a	63.3±11.0b	50.7±4.0c
10^{-10}	5.2±0.2a	57.4±9.0a	45.7±8.5b
10^{-11}	5.8±1.0a	63.4±6.7b	43.5±8.9b
10^{-12}	5.1±0.5a	55.7±6.7a	37.9±6.1b

Values are the mean±standard deviation of 100 clover seedlings in each treatment. Means within the same column and followed by the same letter are not significantly different ($P>0.05$)

Effect of LCOs on Growth and Nodulation of Clover Inoculated with RtKO17-*gusA* Strain and Grown Under Competitive Conditions: The Outdoor Experiment

Finally, we studied the effect of LCO on the growth and nodulation of clover in soil. The clover seeds were soaked in LCOs of the same concentration as in the previous experiment (Table 3) and inoculated with strain RtKO17(pJBA21Tc). This strain was tagged with *gusA* to study its competitiveness in relation to an indigenous population of rhizobia (Table 4). The growth of LCO-treated clover infected by indigenous rhizobia was substantially improved, and the differences in fresh mass of shoots and roots were statistically significant (groups 1 and 2). Also, a 54% increase in total nodule number in relation to untreated seeds was observed. It is worth noting that the growth of clover roots in the presence of LCOs was more robust (1.64-fold) than shoots (1.37-fold) in relation to the untreated control plants.

Growth of LCO-treated clover seeds additionally inoculated with the selected inoculant strain RtKO17-*gusA* was comparable to the untreated control (groups 3 and 4) (Table 4). However, similarly to plants in groups 1 and 2, there was a significant increase in nodule number, about 60%. Generally, a broad variability in nodule number per plant was observed in groups of clover treated with LCOs, independent of the presence of the RtKO17 inoculant (groups 2 and 4), indicating different response of individual plants to LCOs treatment.

To study the effect of LCOs treatment on the competitiveness of RtKO17-*gusA*, we examined nodule occupancy by histochemical staining of whole root systems of clovers 2 and 6 weeks post inoculation (Table 5). After 2 weeks, we found 54% blue nodules in clover untreated with LCOs and 49% Gus^+ nodules formed by plants treated with LCOs. After 6 weeks of cultivation, the proportion of Gus^+ nodules decreased significantly, and strain RtKO17-*gusA* occupied only 14% of nodules in LCO-untreated plants and about 18% of nodules in LCO-treated seeds. These results indicate poor ability of the inoculant strain to compete with autochthonous rhizobial population during prolonged cultivation, unaffected by LCO treatment.

Taken together, the results of the experiment carried out in nonsterile soil revealed the potential of enhancing clover growth and nodulation by LCOs seeds treatment. However, the competitiveness of the selected strain RtKO17-*gusA* in relation to indigenous rhizobia was not enhanced even in seeds stimulated by LCOs.

Discussion

In this study, we describe the promotion of plant growth and nodulation due to treatment of clover seeds with specific Nod factors. In all plant tests, we used LCOs extracted from a culture of strain RtKO17 that had been previously selected as competitive and highly responsive to flavonoid induction as judged by the activation of *nod* genes (Wielbo et al. 2007; Maj et al. 2008). Based on the estimation of LCO biological activity conducted via root hair curling assay, we used a crude preparation of the Nod factor at approximately 10^{-10} M concentration as the most effective. Our study shows that soaking of clover seeds with LCOs RtKO17 causes a statistically significant enhancement of germination and growth of seedlings

Table 3 Effect of LCOs on the growth and nodulation of clover (*T. pratense*) grown in sterile sand and inoculated by RtKO17pJBA21Tc (greenhouse experiment)

Group	Treatment	Fresh mass of shoots (mg)	Fresh mass of roots (mg)	Dry mass of shoots (mg)	Number of nodules/plant
1	Seeds soaked in water	50.5±5.9	10.4±0.9	7.2	–
2	Seeds soaked in LCOs	55.4±7.7	12.2±1.6	7.0	–
3	Seeds soaked in water inoculated with RtKO17	95.4±17.8	21.4±2.8	13.4	2.1±0.4
4	Seeds soaked in LCOs inoculated with RtKO17	109.4±21.8	16.9±4.3	14.6	2.5±0.5

Values are the mean±standard deviation of 50 clover plants in each treatment

Table 4 Effect of LCOs produced by RtKO17 on the growth and nodulation of clover (*T. pratense*) inoculated by RtKO17(pJBA21Tc) grown in soil (outdoor experiment)

Group	Treatment	Fresh mass of shoots (g)	Fresh mass of roots (g)	Dry mass of shoots (g)	Number of nodules/plant
1	Seeds soaked in water	2.12±0.19a	1.68±0.28a	0.3±0.03a	52±13a
2	Seeds soaked in LCOs	2.91±0.31b	2.75±0.43b	0.37±0.04a	83±50a
3	Seeds soaked in water, inoculated RtKO17-*gusA*	2.30±0.39a	1.98±0.31a	0.3±0.05a	57±14a
4	Seeds soaked in LCOs, inoculated RtKO17-*gusA*	2.25±0.20a	2.13±0.31a	0.3±0.06a	76±33a

Values are the mean±standard deviation of 120 clover plants in each treatment. Means within the same column and followed by the same letter are not significantly different ($P>0.05$)

cultivated for 6 weeks in sand under sterile uncompetitive conditions. These results are in agreement with observations reported by Prithiviraj et al. (2003) who showed that LCO Bj V(C18:1,MeFuc) treatment of soybean seeds and nonleguminous plants from diverse botanical families grown for a short time (10 days) under sterile conditions, and also in soil, significantly promoted germination of the seeds and, subsequently, seedlings growth.

The observed increase in the germination of seeds and the growth of clover seedlings after LCOs application could be attributed to an enhanced cell cycle rate described in legumes and nonleguminous plants by several authors (de Jong et al. 1993; Stokkermans et al. 1995; Dyachok et al. 2000). Rhizobial LCOs influenced somatic embryogenesis in Norway spruce (*Picea abies*) and stimulated cell division in Norway spruce protoplasts in the absence of auxin and cytokinin (Dyachok et al. 2000). Nod factors were also shown to stimulate somatic embryogenesis of mutated carrot cell line (de Jong et al. 1993). It was shown that O-acetylated chitin oligosaccharides can induce root cortical cell divisions when these molecules are introduced inside the plant tissue by ballistic microtargeting (Schlaman et al. 1997). There is a possibility that endogenous LCOs analogous in structure to rhizobial Nod factors may be produced in plants (Dyachok et al. 2000).

In the case of RtKO17 inoculation of seeds treated with LCOs and grown in sterile sand, we did not observe a statistically significant increase of fresh and dry mass of shoots (Table 3). The number of nodules elicited by the inoculant strain was also comparable to the untreated control. A possible explanation for the lack of growth promotion could be the excess of Nod factor. In the sterile environment, the factors were also produced by the RtKO17 inoculant strain. The possible excess of Nod factors that are already active in nanomolar concentrations could be due to the lack of autochthonous bacteria that affect the level of Nod factors by production of chitinases capable of factor degradation (Staehelin et al. 1994). Castillo et al. (1999) constructed a derivative of *Sinorhizobium meliloti* strain 1021 with an amplifiable fragment of a symbiotic region, which produced LCOs in higher quantities. A moderate amplification (2.5 copies) of the symbiotic region significantly improved its symbiotic properties, and inoculation of alfalfa with this strain resulted in an enhancement of plant growth. In contrast, higher copy derivatives produced the Nod factor in greater quantities but also reduced nodulation, nitrogen fixation, and plant growth in relation to control plants. These data indicate a tight regulation of *nod* gene expression and are consistent with the previous observation that strains that bear multicopy plasmids containing *nod* genes show reduced nodulation (Knight et al. 1986).

In the outdoor experiment, we tested the possibility of promoting growth and nodulation by LCO treatment of

Table 5 Occupancy of nodules by *R. legumnosarum* bv. *trifolii* strain KO17-*gusA* in the outdoor experiment

Group	Treatment	14days[a]			42days[b]		
		Total no. of nodules	No. of nodules/plant	GUS$^+$ nodules (%)	Total no. of nodules	No. of nodules/plant	GUS$^+$ nodules (%)
1	Seeds soaked in water	364	18±4	0	6,196	52±13	0
2	Seeds soaked in LCOs	396	20±5	0	9,968	83±50	0
3	Seeds soaked in water, inoculated RtKO17-*gusA*	380	19±4	54	5,864	57±14	14
4	Seeds soaked in LCOs, inoculated RtKO17-*gusA*	392	20±4	49	9,084	76±33	18

[a] Values are the mean±standard deviation of 20 clover plants in each treatment

[b] Values are the mean±standard deviation of 120 clover plants in each treatment

clover seeds grown for 6 weeks in nonsterile soil and the competitiveness of the selected strain RtKO17-*gusA* in relation to indigenous rhizobia (Tables 4 and 5). A statistically significant increase of fresh and root clover mass and the percentage of nodule number was the most important effect of LCO seed treatment. An additional inoculation of seeds with RtKO17 did not affect clover growth, but the nodule number increased by approximately 43%, which confirmed LCO stimulation of nodule organogenesis. Conversely, we did not observe any substantial effects of LCO treatment on the competitiveness of RtKO17, which remained low in the treated and untreated control.

Taken together, the effects of Nod factors treatment on seed germination and growth could be explained by a mitogenic effect, thus inducing cortical cell division in roots and leading to the formation of nodules in a compatible plant host (Spaink et al. 1991; Truchet et al. 1991). The most important finding from the presented results is that LCOs pretreatment of seeds before planting could enhance legume growth and nodulation, despite the fact that rhizobia that inhabit the soil are exposed to a mixture of several chemical signals, and the efficiency of nitrogen fixation is determined by the final symbiotic partner.

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Phthalic Acid Induces Oxidative Stress and Alters the Activity of Some Antioxidant Enzymes in Roots of *Malus prunifolia*

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Abstract Apple replant is a widespread agricultural problem documented in all of the major fruit-growing regions of the world. In order to better understand the phytotoxic mechanisms induced by allelochemicals involved with this problem, *Malus prunifolia* plants were grown hydroponically to the six-leaf-stage in the presence of phthalic acid (0 or 1 mM) for 5, 10, or 15 days. Apple plants were evaluated for: shoot and root length, fresh and dry weight, malondialdehyde (MDA) content, hydrogen peroxide (H_2O_2) content, superoxide radical ($O_2^{\cdot-}$) generation rate, and antioxidant enzyme activities. Shoot and root lengths and fresh and dry weights of *M. prunifolia* decreased in plants exposed to phthalic acid. MDA and H_2O_2 content increased in phthalic acid-treated plants as did the generation rate of $O_2^{\cdot-}$ in *M. prunifolia* roots. The activities of superoxide dismutase (EC 1.15.1.1), peroxidase (EC 1.11.1.7), catalase (EC 1.11.1.6), ascorbate peroxidase (EC 1.11.1.11), glutathione reductase (EC 1.6.4.2), dehydroascorbate reductase (EC 1.8.5.1), and monodehydroascorbate reductase (EC 1.6.5.4) increased in phthalic acid-stressed roots compared with control roots. These results suggest that activation of the antioxidant system by phthalic acid led to the formation of reactive oxygen species that resulted in cellular damage and the decrease of *M. prunifolia* growth.

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Introduction

Allelopathy is widely reported in agroecosystem and silviculture, and is implicated in problems such as exotic plant invasion, replant problems, and soil sickness (Lee et al. 2006; Hao et al. 2007; Fernandez et al. 2008). Allelopathic plants exert detrimental effects via the release of plant compounds (allelochemicals) through leaching, root exudation, volatilization, and/or decomposition of plant materials (Weir et al. 2004) and can interfere with the metabolism of other plants. If the effect of such compounds is harmful to plant growth and development, it becomes a biotic or allelochemical stress (Romero-Romero et al. 2005).

Under normal growth conditions, a dynamic equilibrium exists between the production and detoxification of free radicals in cell organelles. Biotic and abiotic stresses may cause the formation of reactive oxygen species (ROS) such as superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot-}$), and/or hydrogen peroxide (H_2O_2) that are commonly generated and accumulated in cells (Cho and Seo 2005). Enhanced ROS production can affect membrane permeability, damage DNA and proteins, induce lipid peroxidation, and ultimately lead to programmed cellular death (Ding et al. 2007). Plants have evolved mechanisms that protect cell and subcellular systems from the effects of ROS by using antioxidant systems such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR), and monodehydroascorbate reductase

(MDHAR). Tolerance to allelochemicals by various crop plants has been associated with an increase in the activity of antioxidant enzymes (Yu and Matsui 1997).

The apple replant problem is widespread. Studies have shown that allelochemicals in root exudates or decomposition of residues may play a role in this agricultural problem (Borner 1959; Zhang et al. 2007; Bai et al. 2009). However, the mechanisms involved have not been investigated extensively. Reports suggest that allelochemicals cause increased membrane leakage, and enhance H_2O_2 and MDA levels in the target plant tissues. For instance, cinnamic acid enhanced the generation of free radicals, increased lipid peroxidation, and oxidative membrane damage in cucumber and figleaf gourd plants (Ding et al. 2007). Similarly, 2-benzoxazolinone inhibited lettuce growth, caused membrane damage, and increased MDA and H_2O_2 production (Sanchez-Moreiras and Reigosa 2005). Most studies in this regard focused on vegetables and agronomic crops (Baziramakenga et al. 1995; Yu et al. 2003; Ye et al. 2006). However, it is not known whether the inhibitory effects were mediated through production of ROS in apple roots under allelochemical stress. Phthalic acid is a potent allelochemical and inhibits the growth of a number of plants such as *Lactuca sativa* L. (Lee et al. 2006), *Zea mays* L. (Chai and Feng 2007), and *Malus prunifolia* (Bai et al. 2009). Accordingly, its effect on plant growth, ROS generation rate, and ROS-antioxidant enzyme activity were investigated in *M. prunifolia* seedlings.

Methods and Materials

Plant Materials and Phthalic Acid Treatment Seeds of apple rootstock, *M. prunifolia*, were obtained from Fuping County (34°75′ N, 109°15′ E), Shaanxi Province. Seed sterilization was done according to Zhang et al. (2007) and involved surface-sterilization in 0.3% (*v*/*v*) H_2O_2 for 20 min, followed by several rinses with sterile H_2O. The sterilized seeds were stratified at 4°C for 85 days. Sprouted seeds were sown in plastic pots (9 cm in diameter, 12 cm high; three seeds per pot) filled with sterilized sand. All pots were placed in a greenhouse at the College of Horticulture, Northwest A&F University, Yangling (34°20′ N, 108°24′ E). Plants were grown without supplementary illumination with night and day temperatures at 20 to 25°C and relative humidity at 65–80%. Seedlings were watered once a week with Hoagland nutrient solution (Hoagland 1920), pH 6.0±0.2. When the seedlings reached the six-leaf-stage, batches of 45 uniform seedlings were transferred into a hydroponic system (plastic container; 45×37×22.5 cm) filled with 5 l half Hoagland nutrient solution at pH 6.0±0.2 and electrical conductivity at 1.2 ms/cm, respectively. The containers were placed in a controlled growth room with a L/D regime of 12/12 h, 25/20°C, and a photon flux density of 140–160 μmol m^{-2} s^{-1}. Seedlings were allowed to acclimate to the hydroponic conditions for 5 days. Phthalic acid (purchased from Yifang S&T Ltd. Tianjin, China), dissolved in ethanol, was added to the nutrient solution to concentrations of 0 or 1 mM. In the preliminary experiment, thinner *M. prunifolia* leaves and brownish root apices in parallel with a large amount of mucilage was secreted from roots 15 days after application of 1 mM phthalic acid. This indicates that phthalic acid at this concentration is lethal to *M. prunifolia* seedlings. The final concentration of ethanol in control and treatment solutions was 0.1% (*v*/*v*). The nutrient solutions in the containers were aerated continuously by air pumps. The solution in the plastic container was kept at the same level by adding half Hoagland nutrient solution at 24 h intervals. Each treatment was replicated three times in a completely randomized design. Root samples were taken from both control and phthalic acid-treated plants on days 5, 10, and 15 after treatment, and the tissue was frozen in liquid nitrogen and stored at −70°C until analysis. At the end of the experiment (15 days after treatment), root and shoot length and fresh and dry weight of seedlings were measured. The seedlings were dried in an forced-air oven at 60°C until constant mass was reached.

Measurement of MDA, H_2O_2, and $O_2^{\cdot-}$ Generation Lipid peroxidation was followed by measuring MDA accumulation using the method of Baziramakenga et al. (1995) with some modifications. Root samples (0.2 g) were homogenized in 0.1% of trichloroacetic acid in phosphate buffer (5 ml; pH 7.8) and centrifuged at 12,000×*g* for 15 min. Supernatant (1 ml) was added to 0.5% thiobarbituric acid in 20% trichloroacetic acid (4 ml). The mixture was placed in a water bath at 100°C for 10 min and then quickly cooled in an ice-bath for 15 min. Samples were centrifuged at 12,000×*g* for 5 min, and then the absorbance of the supernatant was measured at 450, 532, and 600 nm.

H_2O_2 in the supernatant was measured according to Patterson et al. (1984). Root samples (0.5 g) were homogenized in 5-ml pre-cooled acetone and centrifuged for 10 min at 1,500×*g*. Titanium chloride (0.1%, *w*/*v*) and concentrated ammonia (0.2 ml) were added into the supernatant (1 ml), the mixture was allowed to react (10 min at 25°C) and the reaction mixture was centrifuged at 1,500×*g* for 10 min. Absorbance at 410 nm was measured, and the H_2O_2 concentration was calculated according to a standard curve.

The rate of $O_2^{\cdot-}$ generation was measured as described by Elstner and Heupel (1976) with some modifications. Root tissue (1 g) was homogenized in 65 mM potassium phosphate buffer (3 ml; pH 7.8). The homogenate was centrifuged at (10,000×*g* for 15 min). The supernatant

(0.5 ml) was added to 65 mM potassium phosphate buffer (0.5 ml; pH 7.8) containing 10 mM hydroxylammonium-chloride (0.1 ml) and incubated (25°C for 20 min). Sulphanilic acid (58 mM; 1 ml) and α-naphthyl amine (7 mM; 1 ml) were added to the mixture, and it was allowed to incubate (25°C for 20 min). The final solution was mixed with an equal volume of chloroform and the absorbance of the pink phase was measured at 530 nm.

Extraction and Assay of Enzyme Activities Antioxidant enzymes (SOD, POD, and CAT) were extracted according to the method of Yu et al. (2003) with some modifications. Root samples (0.5 g) were homogenized in phosphate buffer (8 ml; 0.1 M; pH 7.5) containing 2% (*w*/*v*) polyvinylpyrrolidone. The homogenate was centrifuged (12,000×*g* for 20 min) and the supernatant was used for enzyme analysis. All assays were carried out at 2–4°C.

Superoxide dismutase activity was measured according to Beauchamp and Fridovich (1971) with minor modification. The assay medium (3 ml) contained phosphate buffer (50 mM; pH 7.8), EDTA-Na (0.1 mM), L-methionine (12 mM), riboflavin (2 μM), and nitrotetrazolium blue chloride (75 μM). Riboflavin was added last. The tubes were shaken and placed at a photosynthetic photon flux of 50 μmol m^{-2} s^{-1} for 15 min. The reaction was initiated and terminated by turning the light on and off, respectively. The A_{560} was measured on a spectrophotometer and tubes containing the assay mixture, but without the root extract (control), were illuminated to determine maximum A_{560}.

Peroxidase activity was measured according to Sofo et al. (2004) with some modification. The reaction solution (3 ml) contained phosphate buffer (2.9 ml, 50 mM; pH 7.0), guaiacol (50 μl; 10 mM), H_2O_2 (10 μl; 40 mM), and crude enzyme extract (40 μl). The Increase in A_{470nm} due to the oxidation of guaiacol was measured at 20°C.

Catalase activity was assayed by monitoring the decrease in A_{240nm} (Aebi 1984). The reaction mixture contained phosphate buffer (50 mM; pH 7.0) and H_2O_2 (30% *w*/*v*) and was started by adding the reaction solution to crude extract (10 μl).

AsA-related enzymes were extracted according to Nakano and Asada (1981). Generally, each 0.5 g of roots material was homogenized in KH_2PO_4-KOH (6 ml, 50 mM; pH 7.5) containing ethylenediaminetetraacetic acid (0.1 mM), Triton X-100 (0.3% *v*/*v*), and insoluble polyvinylpolypyrrolidone (4% *w*/*v*). The homogenate was centrifuged (16,000×*g* for 15 min at 2°C) and the supernatant was used for APX, GR, MDHAR, and DHAR analyses.

Ascorbate peroxidase was measured by monitoring the decrease in $A_{290\ nm}$ (Nakano and Asada 1981). The assay mixture (1 ml) contained Hepes–KOH (50 mM; pH 7.6), ethylenediaminetetraacetic acid (0.1 mM), H_2O_2 (0.2 mM, AsA 0.5 mM), and enzyme extract. The reaction was initiated by adding H_2O_2.

Glutathione reductase activity was monitored at $A_{340\ nm}$ in a 1 ml reaction mixture containing Tris–HCl (100 mM; pH 8.0), ethylenediaminetetraacetic acid (1 mM), oxidized glutathione (GSSG;1 mM), and NADPH (0.2 mM). The reaction was initiated by adding NADPH (Grace and Logan 1996).

Monodehydroascorbate reductase activity was assayed at 340 nm in a 1 ml reaction mixture containing Hepes–KOH (50 mM; pH 7.6), NADH (0.1 mM), AsA (0.25 mM), and AsA oxidase (EC 1.10.3.3; 0.25 U). The reaction was initiated by adding AsA oxidase (Miyake and Asada 1992).

Dehydroascorbate rductase activity was measured at 265 nm in a 1 ml assay solution containing Hepes–KOH (100 mM; pH 7.0), ethylenediaminetetraacetic acid (1 mM), GSH (2.5 mM), and DHA (0.2 mM). The reaction was initiated by adding DHA (Dalton et al. 1986).

Statistical Analysis All data were subjected to analysis of variance, followed by Tukey's Studentized Range Test (SAS Statistical package, version 8.2). Results are presented as the means±standard deviation (SD).

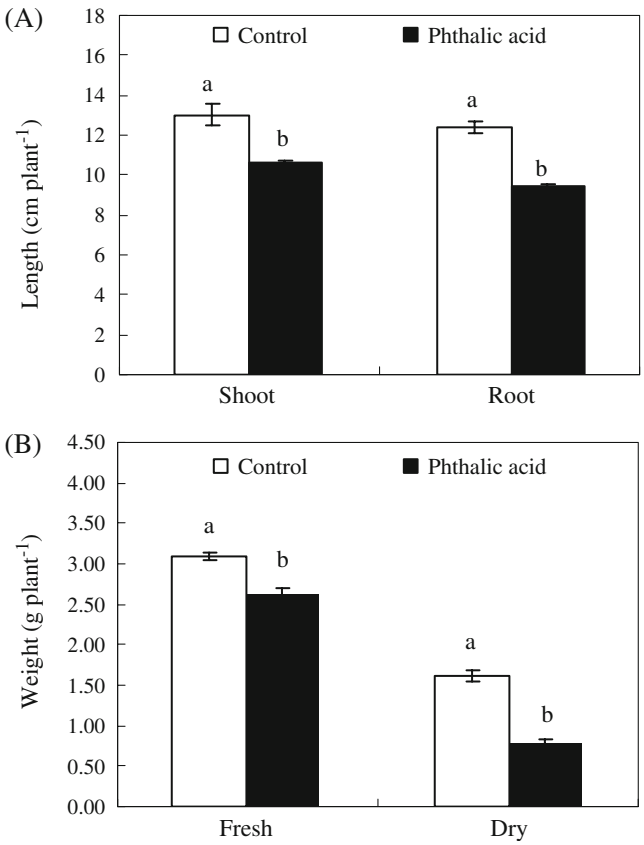

Fig. 1 Shoot and root length (**a**) and fresh and dry weight (**b**) of *Malus prunifolia* plants grown at 0 (control) or 1 mM phthalic acid. Samples were taken 15 days after treatment. Values are means of three replicates± standard error (SE). Significant difference ($P<0.05$ level) was tested between the control and 1 mM phthalic acid treatment for each dependent variable and indicated by different *letters above the bars*

Results

Effect on Plant Growth The toxic effect of 1 mM phthalic acid appeared after 15 days following treatment. The length of shoot and root of *M. prunifolia* plants had reduced shoot and root length compared to lower than controls (Fig. 1a). Fresh and dry weights were 3.09 and 1.61 g plant^{-1} in control plants compared to 2.63 and 0.79 g plant^{-1} for plants treated with 1 mM phthalic acid (Fig. 1b).

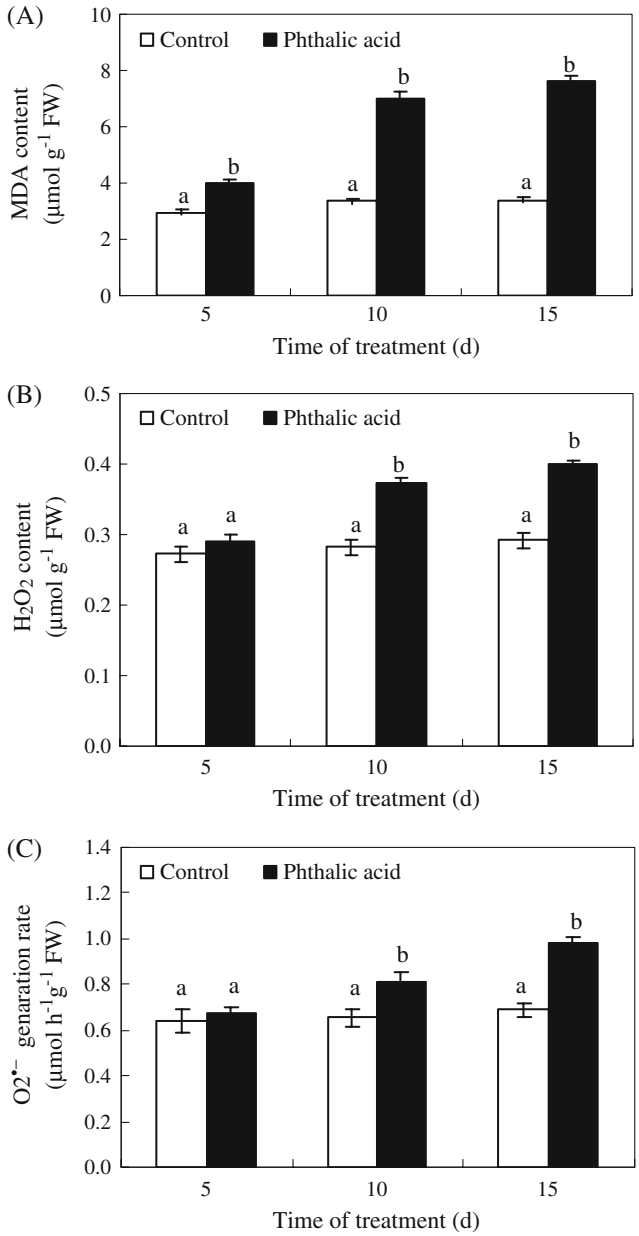

Fig. 2 MDA (**a**), H_2O_2 (**b**) content, and $O_2^{\cdot-}$ generation rate (**c**) in *Malus prunifolia* plants grown at 0 (control) or 1 mM phthalic acid. Values are means of three replicates±standard error (SE). Significant difference ($P<0.05$ level) was tested between the control and 1 mM phthalic acid treatment at each sampling date separately and indicated by different *letters above the bars*

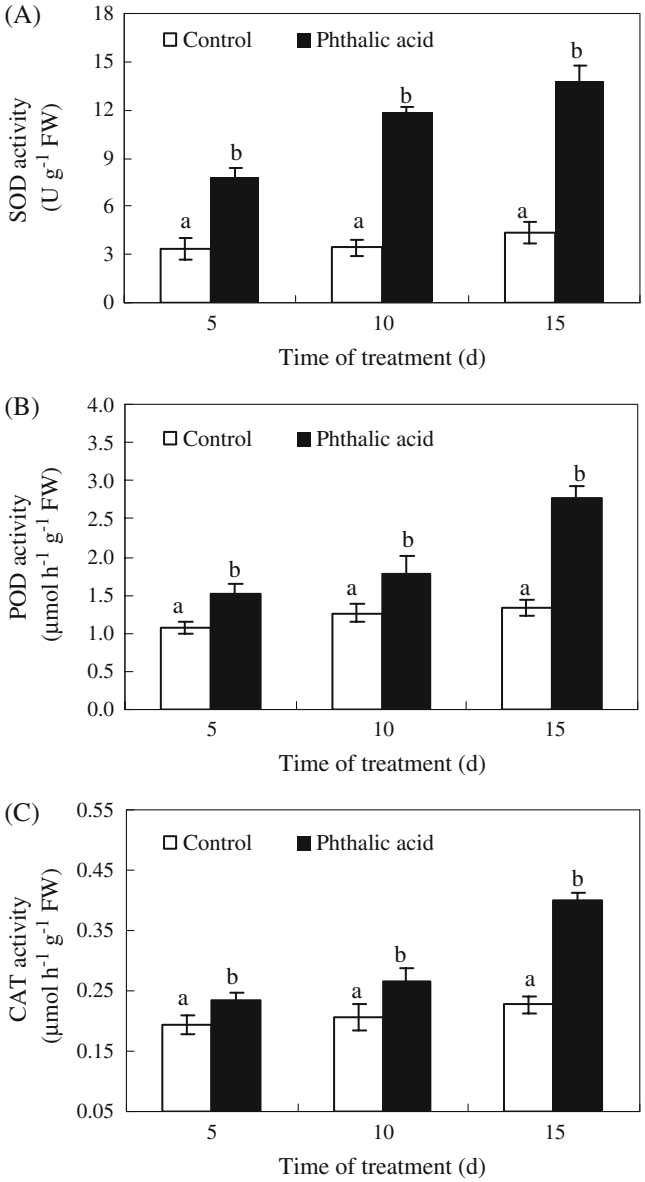

Fig. 3 SOD (**a**), POD (**b**), and CAT (**c**) activities of *Malus prunifolia* plants grown at 0 (control) or 1 mM phthalic acid. The values are means of three replicates±standard error (SE). Significant difference ($P<0.05$ level) was tested between the control and 1 mM phthalic acid treatment at each sampling date separately and indicated by different *letters above the bars*

Effect on MDA, H_2O_2, and $O_2^{\cdot-}$ Generation MDA concentration in *M. prunifolia* roots increased significantly after 5, 10, and 15 days of treatment (Fig. 2a). The maximum increase of 125.4% was observed after 15 days. Exposure to phthalic acid also resulted in an increase in the H_2O_2 content and $O_2^{\cdot-}$ generation rate, respectively (Fig. 2b, c). The maximum increases of 37.2% and 29.9%, respectively, were observed after 15 days.

Effect on Enzyme Activity In response to phthalic acid, activities of SOD, POD, and CAT, as well as enzymes in

ascorbate-glutathione cycle, e.g., APX, GR, MDHAR, and DHAR, all showed similar trends in the activity of root enzymes in response to phthalic acid. In general, enzyme activities increased progressively during the treatment period (days 5–15) compared to enzymes in the roots of plants not exposed to phthalic acid (Figs. 3 and 4).

Discussion

Phthalic acid treatment significantly decreases the growth of *M. prunifolia* plants (Fig. 1). This finding is in agreement with studies on *L. sativa* L. (Lee et al. 2006) and *Z. mays* L. (Chai and Feng 2007), and consistent with the inhibitory effects of other allelochemicals on plant growth reported previously (Lin and Kao 2000; Asao et al. 2004; Batish et al. 2008).

The formation of free radials in cells results in damage to cell membranes due to lipid peroxidation. Thus, the level of MDA, produced during lipid peroxidation, is a good indicator of oxidative damage that could be occurring within cells (Masia 2003). MDA accumulation due to lipid peroxidation has been reported in response to a variety of abiotic and biotic stresses (Dhindsa et al. 1981; Apel and Hirt 2004). In the present study, enhanced MDA (Fig. 2a) suggests that phthalic acid probably induces oxidative stress, and, as a result, disrupts cellular membrane structure, and causes a loss of cellular integrity. Similar results with other allelochemicals have been found (Wu et al. 2002; Batish et al. 2006; Ye et al. 2006). Many studies show that increased MDA content is associated with increased $O_2^{\cdot-}$ and H_2O_2 production following biotic and abiotic stresses (Forman et al. 2002; Lara-Nunez et al. 2006). Here, phthalic acid induced $O_2^{\cdot-}$ and H_2O_2 production in *M. prunifolia* roots (Fig. 2b, c), which suggests that phthalic acid could trigger ROS generation and induce oxidative stress in *M. prunifolia* roots. This observation is consistent with that obtained with mung bean treated with 2-benzoxazolinoen (Batish et al. 2006). Increases in SOD activity (Fig. 3a) also indicate that excessive generation of $O_2^{\cdot-}$ has been triggered by pthalic acid treatment, and, consequently, that SOD activity was up-regulated to mitigate the oxidative damage. Similar responses have been

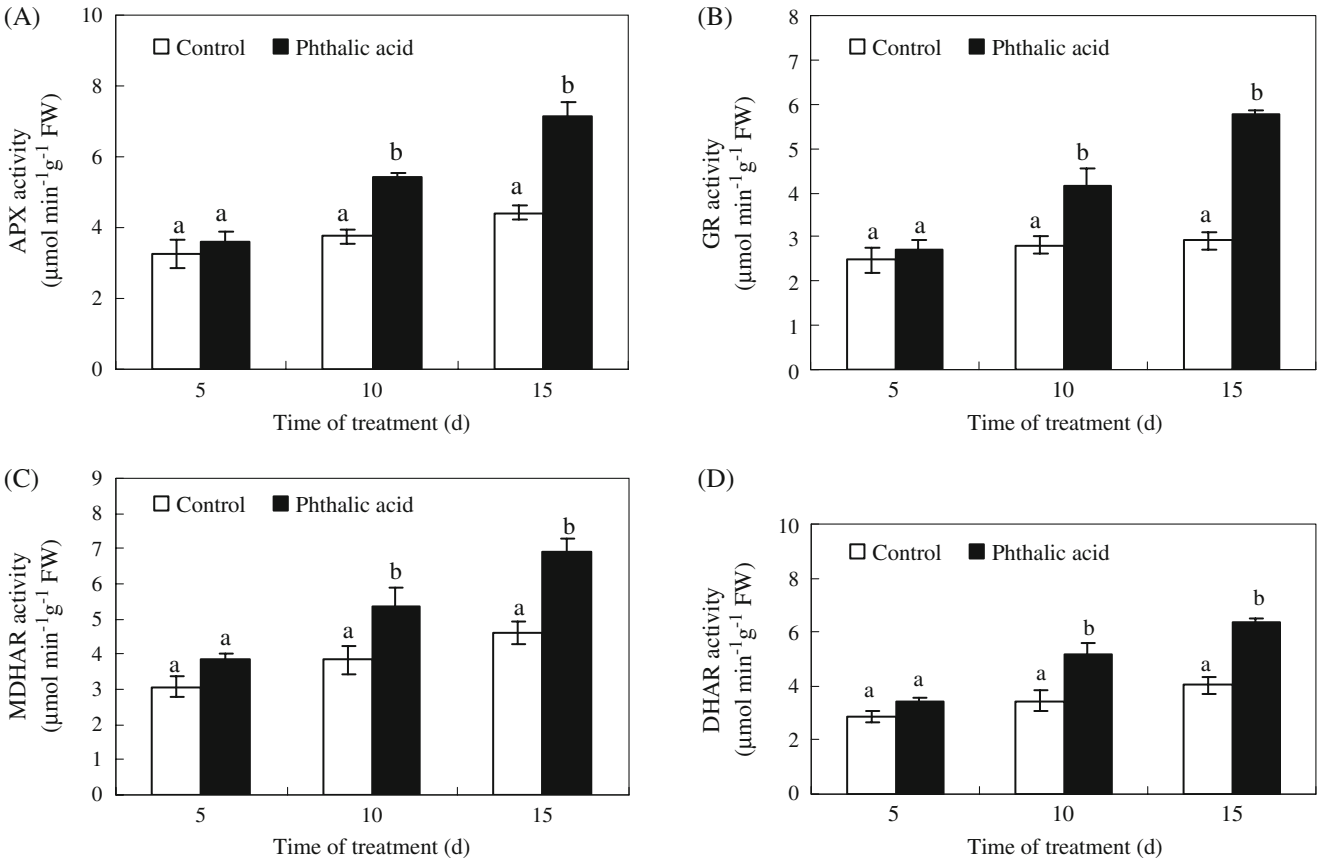

Fig. 4 APX (**a**), GR (**b**), MDHAR (**c**), and DHAR (**d**) activities of *Malus prunifolia* plants grown with 0 (control) or 1 mM phthalic acid. The values are means of three replicates±standard error (SE). Significant difference ($P<0.05$ level) was tested between the control and 1 mM phthalic acid treatment at each sampling date separately and indicated by different *letters above the bars*

observed in plants treated with other allelochemicals (Yu et al. 2003; Batish et al. 2006; Wu et al. 2002; Lin et al. 2007).

Accumulation of H_2O_2 in *M. prunifolia* roots in response to phthalic acid treatment enhances lipid peroxidation and causes a severe oxidative stress resulting in disruption of metabolic activities in the cell. Enhanced H_2O_2 levels are removed by the antioxidant enzymes such as CAT, APX, and POD (Blokhina et al. 2003), and glutathione-ascorbate cycle (Nakano and Asada 1981). In the present study, increases in the activities of these antioxidant enzymes paralleled the accumulation of MDA and H_2O_2 in *M. prunifolia* root after exposure to phthalic acid (Figs. 2–4). Our observations are consistent with those of Cruz-Ortega et al. (2002), who reported that allelochemical stress causes increases in the level of free radicals and the activity of antioxidant enzymes and suggests that increased induction of these enzymes is necessary to prevent lipid peroxidation (i.e., to counter the higher MDA and H_2O_2 in roots). Although there were increased levels of antioxidants in roots after exposure to phthalic acid, oxidative stress still occurred. Our data are consistent with those of Batish et al. (2006), who showed that exposure of mung bean to 2-benzoxazolinone increased APX, GR, and CAT activity. An increased POD activity in response to allelochemicals also has been demonstrated in cucumber root (Yu et al. 2003). Oracz et al. (2007) observed an increase in the cell membrane permeability, MDA level, H_2O_2 concentration, and SOD and CAT activity in mustard treated by sunflower extract. Furthermore, the activity and expression of most antioxidant enzymes is stimulated by ROS accumulation (Apel and Hirt 2004).

In summary, phthalic acid induces oxidative stress in *M. prunifolia* roots through the generation of ROS and decreases plant growth, despite the concomitant increase in antioxidant enzymes. This might be one of the mechanisms responsible for the apple replant problem. The increase in antioxidant enzymes could reflect a defensive response to the cellular damage provoked by phthalic acid treatment. However, this increase was not strong enough to eliminate all the deleterious effects provoked by phthalic acid, only alleviated the impact of stress.

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Density-Dependent Phytotoxicity of *Impatiens pallida* Plants Exposed to Extracts of *Alliaria petiolata*

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Abstract Invasive plants are by definition excellent competitors, either indirectly through competition for resources or directly through allelopathic inhibition of neighboring plants. Although both forms of competition are commonly studied, attempts to explore the interactions between direct and indirect competition are rare. We monitored the effects of several doses of extracts of *Alliaria petiolata*, a Eurasian invader in North America, on the growth of *Impatiens pallida*, a North American native, at several planting densities. The density-dependent phytotoxicity model predicts that as plant density increases, individual plant size will decrease, unless a toxin is present in the soil. In this case, individual plant size is predicted to increase as plant density increases, as plants share a limited toxin dose. We tested this model using fractions of an *A. petiolata* extract enriched in flavonoids or glucosinolates, as well as a combined fraction. The flavonoid-enriched fraction and the combined fraction suppressed *I. pallida* growth but only when applied at a dose eight times higher than that expected in the field. When treated with a dose equivalent to estimated field exposure levels, *I. pallida* growth was not distinguishable from that of control plants that received no extract, showing that indirect competition for resources was more important for determining the growth of *I. pallida* than direct allelopathic inhibition by *A. petiolata*. This is an important reminder that, even though many plants have the demonstrated potential to exert strong allelopathic effects, those effects may not always be apparent when other forms of competition are considered as well.

Keywords Allelopathy · Invasion · Glucosinolates · Flavonoid glycosides

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Introduction

In natural communities, plants compete in complex ways as they vie for the same territory and resources. These interactions can be classified as direct or indirect competition and are difficult to distinguish experimentally since they operate in concert. Indirect competition is widely assumed to be the default natural condition, where two plants require access to a limited resource and the "winner" is more efficient at acquiring that resource (Connell 1990). Direct competition occurs through interference, with allelopathy being one of the most common mechanisms. Allelopathic plants release compounds into the environment that negatively impact surrounding plants, thus giving the allelopath a competitive advantage (Rice 1974).

Invasive plant species are by definition excellent competitors, whether by direct competition, indirect competition, or both. The distinction is important for several reasons. Simply removing the invader may control the spread of an invasion by indirect competition. However, in a site where direct competition is occurring, removal of the invader may not be enough. Allelochemicals released by the invader could remain in the soil and continue to inhibit

growth of other plants, even after removal of the allelopathic invader. For this reason, considerable attention has been paid to exploring the competitive mechanisms of invasive plants (Levine et al. 2004; Theoharides and Dukes 2007), including *Alliaria petiolata* (M. Bieb.) Cavara and Grande, Brassicaceae (garlic mustard; Meekins and McCarthy 1999; Bauer et al. 2005).

A. petiolata, an herbaceous biennial, was introduced into North America from Europe in the 1860s and has since become invasive in much of Canada and the northeast and Midwestern USA (Nuzzo 2002). The success of *A. petiolata* in North America has been attributed to its high propagule pressure (Cavers et al. 1979), escape from herbivores (Blossey et al. 2001), superior competitive ability (Meekins and McCarthy 1999), and allelopathic inhibition of surrounding plants (Vaughn and Berhow 1999; Roberts and Anderson 2001; Prati and Bossdorf 2004; Stinson et al. 2006; Wolfe et al. 2008). Given the broad range of bioactive secondary metabolites produced by garlic mustard (Haribal and Renwick 1998, 2001; Vaughn and Berhow 1999; Haribal et al. 2001; Renwick et al. 2001; Cipollini and Gruner 2007), it is not surprising that a large part of the effort spent studying the invasive success of this species has focused on allelopathic effects. Crude extracts and purified compounds from *A. petiolata* inhibited germination and growth of other plants and their symbiotic arbuscular mycorrhizal fungi (Vaughn and Berhow 1999; Roberts and Anderson 2001; Stinson et al. 2006; Callaway et al. 2008). Seeds of *Geum* spp. sown in soil conditioned by *A. petiolata* germinated better if the soil also contained activated carbon, presumably indicating the presence of inhibitory organic compounds in the soil (Prati and Bossdorf 2004). Cipollini et al. (2008b) also found positive effects of adding activated carbon on growth of *Impatiens capensis* growing with *A. petiolata* in the field. In contrast, *A. petiolata* extracts have failed to inhibit germination or growth of several plant species in some studies (McCarthy and Hanson 1998; Cipollini et al. 2008a). While *A. petiolata* clearly has the potential to act allelopathically, whether allelopathic effects are important in more realistic environments where resource competition is also occurring is largely unknown.

Activated carbon has been advocated for use as a soil amendment as a simple way to distinguish between allelopathy and resource competition because it should adsorb organic compounds with little effect on inorganic nutrients (Inderjit and Callaway 2003). However, it can alter nutrient levels in unpredictable ways (Lau et al. 2008), and other methods to verify the allelopathic potential of plant species are therefore necessary. Allelopathic effects are highly dependent on densities of target species and can be masked by resource competition at high densities (Weidenhamer et al. 1989; Weidenhamer 1996). According to the density-dependent phytotoxicity model, if resource competition is the dominating factor in an interaction, then individual plant mass will decrease as density increases. However, if allelopathy is the dominant factor, then individual plant mass will decrease more slowly, or even increase, as density increases, until a density is reached where resource competition among target plants becomes the dominant factor (Fig. 1). This model assumes that all plants present must share the available dose of an allelochemical, and as plant density increases the dose per plant decreases. At very high densities, each plant receives a dose so low that allelopathic inhibition disappears, and resource competition among target plants thus becomes dominant (Weidenhamer et al. 1989). This pattern has been observed for plants exposed to purified compounds (Hoffman and Lavy 1978; Andersen 1981; Weidenhamer et al. 1989), ground tissue from suspected allelopaths (Tseng et al. 2003), and soil conditioned by an allelopathic plant (Weidenhamer et al. 1989).

Allelopathic effects of *A. petiolata* may be masked by resource competition among target plants in some cases, and a better understanding of the dose–response relationship of secondary metabolites of *A. petiolata* would help clarify this issue. Synergism among allelochemicals also may be contributing to the inconsistency of results reported in the literature since fractions of extracts and even purified compounds have been used (Vaughn and Berhow 1999; Roberts and Anderson 2001). The objective of this experiment was to use a density-dependent phytotoxicity approach to determine the allelopathic potential of *A. petiolata* for a range of extract doses. We used fractionated extracts, enriched in either glucosinolates or flavonoid

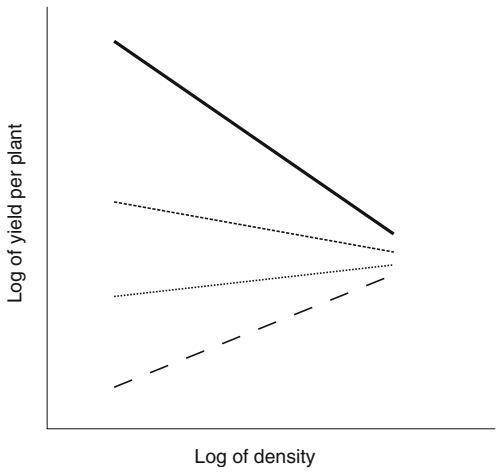

Fig. 1 Predictions of the density-dependent phytotoxicity model. The *bold line* represents expectations when resource competition is dominant, and the *dashed line* represents expectations when allelopathy is dominant. *Dotted lines* represent expected patterns at intermediate chemical doses

glycosides, to explore the contribution of different classes of compounds to observed allelopathic effects.

Methods and Materials

We chose *I. pallida* (Nutt.) Balsaminaceae (pale jewelweed) as the target plant for this study because it grows in the same habitats invaded by *A. petiolata* and has been susceptible to allelopathic effects of *A. petiolata* in prior studies (Barto, unpublished data). *I. pallida* seeds were collected from a population in Yellow Springs, OH, USA (39° 47.0' N, 83° 52.5' W), and nonsterilized seeds were stratified immediately in sterile water at 3°C to stimulate germination. Then, seedlings were transferred to 15-cm (1 L) pots containing sieved field soil mixed 1:1 with sand. The experiment was fully factorial with four densities (one, two, four, and eight *I. pallida* plants), three *A. petiolata* fractions (glucosinolate-enriched fraction, flavonoid-enriched fraction, and a combined fraction), and six concentrations (0.25*X*, 0.5*X*, 1*X*, 2*X*, 4*X*, and 8*X*) with *X* equaling 3.3-mg tissue equivalents per gram soil. This dose was chosen to represent expected exposure levels in the field (Callaway et al. 2008). A set of controls that received only water was planted at all four densities, and at least three replicate pots were planted for each treatment combination.

Extracts were prepared and fractionated, as in Callaway et al. (2008). We boiled *A. petiolata* tissues in ethanol, then filtered and dried the extract before defatting with hexane to remove chlorophylls. We wanted to focus on the glucosinolates and flavonoids, so the hexane fraction was discarded, even though it may have contained lipophilic

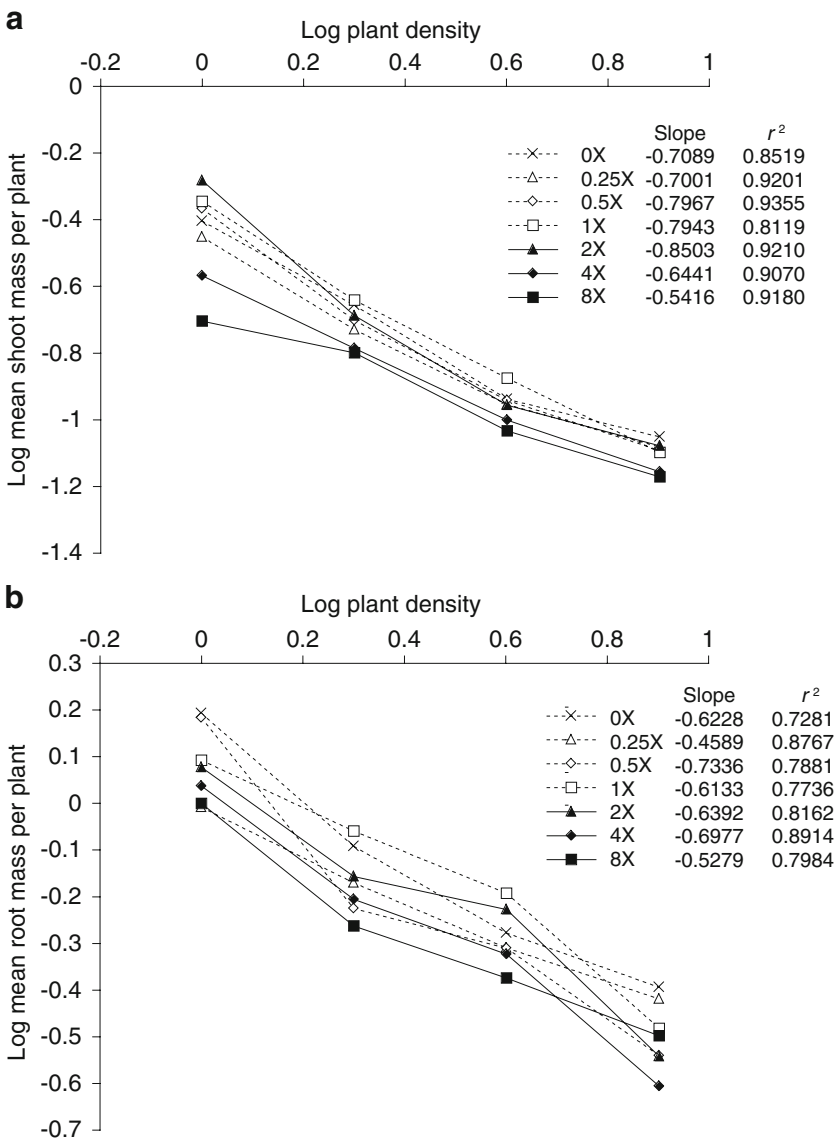

Fig. 2 Response of *Impatiens pallida* plants exposed to a flavonoid-enriched fraction of an *Alliaria petiolata* extract (*N*=2–3). **a** Relationship of log mean shoot mass per plant and log plant density. **b** Relationship of log mean root mass per plant and log plant density

allelochemicals. We dissolved the dried extract in water, then partitioned with *n*-butanol to separate the flavonoid glycosides and alliarinoside from the glucosinolates (Callaway et al. 2008). Both extracts were dried and redissolved in water before use. Pots were dosed weekly and watered as needed. Plants were harvested after 6 weeks and air-dried at 25°C for 1 week, and shoot and root dry masses were measured. Root-to-shoot ratios were calculated, and the log of root and shoot dry mass per plant and log of root-to-shoot ratios were regressed against the log of plant density per pot using PROC GLM with density and dose as factors, followed by Duncan's multiple-range test with α=0.05 (Weidenhamer et al. 1989). SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) was used for all analyses.

Results

Across all other factors, mean shoot dry masses were higher in pots dosed with a glucosinolate-enriched fraction than those that received either a combined extract or a flavonoid-enriched fraction ($F_{2,\ 161}$=3.55, P=0.031). Mean root dry masses showed a similar pattern, and the effect was almost significant at the P=0.05 level ($F_{2,\ 161}$=2.88, P=0.059). Mean root and shoot mass per plant declined with increasing plant density at every dose.

Among pots dosed with a flavonoid-enriched fraction, plant density was the most significant factor that impacted shoot dry mass (t=−19.07, P<0.001; Fig. 2a, Table 1). Dose was also significant (t=−4.98, P<0.001), but shoot mass after exposure to the 8*X* dose was lower than that of control plants only at the lowest density. This suggests that, at densities greater than one plant per pot, resource competition among *I. pallida* plants was masking any allelopathic effects of *A. petiolata* extracts. The slopes of the regression lines were significantly different (t=2.16, P=0.034), supporting predictions of the density-dependent phytotoxicity model. However, the slope for the 1*X* dose could not be distinguished from that of the control, indicating that, at an ecologically relevant dose, allelopathic inhibition was not occurring. Plant density also was the most significant factor impacting root dry mass (t=−12.81, P<0.001) of plants exposed to a flavonoid-enriched fraction, but dose had no effect (t=−1.90 P=0.068; Fig. 2b, Table 1). Root dry mass was not affected by a flavonoid-enriched fraction since the slopes of the regression lines were not significantly different (t=0.26, P=0.797).

Effects were much less pronounced in pots dosed with a glucosinolate-enriched fraction. Plant density was again the most significant factor that affected mean shoot and root dry masses (shoot: t=−11.94, P<0.001; root: t=−14.02, P< 0.001; Fig. 3, Table 2). Dose impacted mean shoot dry masses (t=2.13, P=0.036), while mean root dry masses were unaffected (t=−0.25, P=0.802). As before, the slopes of the regression lines were not significantly different in either case (shoot: t=−0.17, P=0.866; root: t=1.13, P= 0.264), suggesting that resource competition among *I. pallida* plants was the dominant factor when they were exposed to glucosinolates alone.

Inhibitory effects were most pronounced in pots dosed with both flavonoid- and glucosinolate-enriched fractions, representing a more complete phytochemical profile of *A. petiolata*. Plant density again was the most significant factor

Table 1 Shoot and root dry masses of *I. pallida* plants exposed to a flavonoid-enriched fraction of an *A. petiolata* extract

Dose	One plant per pot	Two plants per pot	Four plants per pot	Eight plants per pot
Mean shoot dry mass per plant (mg±1 SE)[a]				
0*X*	394±93 abc	221±11 de	115±8 ghi	89±5 ijh
0.25*X*	353±42 bc	186±12 def	111±10 ghi	83±8 ij
0.5*X*	429±46 ab	199±23 def	114±1 ghi	81±5 ij
1*X*	452±114 ab	229±67 de	133±15 fgh	80±8 ij
2*X*	522±50 a	205±17 de	111±3ghi	84±5 ij
4*X*	270±46 cd	164±19 efg	100±3 ijh	70±4 j
8*X*	198±14 def	159±2 efg	93±7 ijh	67±10 j
Mean root dry mass per plant (mg±1 SE)[a]				
0*X*	1,557±320 a	808±188 bcdefg	527±90 fghijkl	404±27 ijklm
0.25*X*	980±8 abcd	675±80 cdefghi	490±49 ghijkl	381±36 jklmn
0.5*X*	1,524±318 a	595±110 efghij	490±15 ghijkl	288±50 mn
1*X*	1,236±165 ab	871±173 bcdef	641±167 defghij	329±20 klmn
2*X*	1,193±290 ab	697±62 cdefgh	592±67 defghij	287±42 mn
4*X*	1,088±77 abc	623±28 defghij	475±27 ghijkl	248±54 n
8*X*	999±239 abcde	546±29 fghijk	422±39 hijklm	317±23 lmn

In dose column, *X* represents expected field levels of 3.3 mg *A. petiolata* tissue equivalents per gram soil

[a] Means with the same letter are not significantly different at α= 0.05 using Duncan's multiple-range test

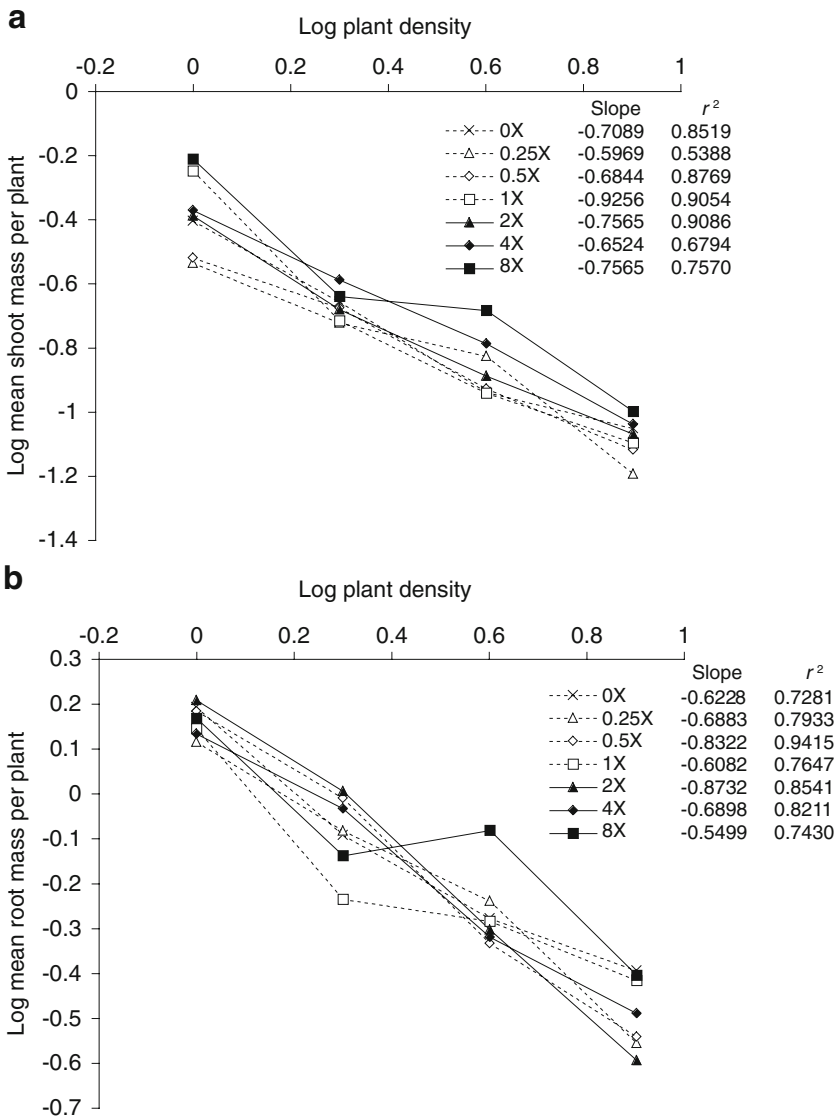

Fig. 3 Response of *I. pallida* plants exposed to a glucosinolate-enriched fraction of an *A. petiolata* extract (N=2–4). **a** Relationship of log mean shoot mass per plant and log plant density. **b** Relationship of log mean root mass per plant and log plant density

Table 2 Shoot and root dry masses of *I. pallida* plants exposed to a glucosinolate-enriched fraction of an *A. petiolata* extract

Dose	One plant per pot	Two plants per pot	Four plants per pot	Eight plants per pot
Mean shoot dry mass per plant (mg±1 SE)[a]				
0*X*	394±93 abc	221±11 bcdefgh	115±8 hijklm	89±5 jklm
0.25*X*	291±106 bcdefg	189±27 defghi	149±22 fghijk	64±2 m
0.5*X*	302±43 abcde	211±10 bcdefgh	118±14 hijklm	76±15 lm
1*X*	564±85 a	192±27 cdefghi	114±8 hijklm	80±9 klm
2*X*	409±18 ab	209±43 cdefgh	130±4 ghijkl	85±13 jklm
4*X*	426±139 abcd	258±21 bcdef	163±3 efghij	92±11 jklm
8*X*	615±216 a	229±45 bcdefgh	208±3 cdefgh	100±9 ijklm
Mean root dry mass per plant (mg±1 SE)[a]				
0*X*	1,557±320 a	808±188 cdefgh	527±90 ghij	404±27 ijklm
0.25*X*	1,304±336 abcd	827±77 bcdefg	576±76 fghij	278±22 lm
0.5*X*	1,526±98 a	977±51 abcdef	464±68 hijkl	287±33 klm
1*X*	1,397±14 ab	582±61 efghi	520±132 ghijk	384±56 ijklm
2*X*	1,615±421 a	1,013±53 abcde	498±39 ghijk	255±67 m
4*X*	1,359±257 abc	926±86 abcdef	480±80 ghijkl	324±31 jklm
8*X*	1,472±301 ab	728±88 defgh	829±137 bcdefg	394±24 ijklm

In dose column, *X* represents expected field levels of 3.3 mg *A. petiolata* tissue equivalents per gram soil

[a] Means with the same letter are not significantly different at α= 0.05 using Duncan's multiple-range test

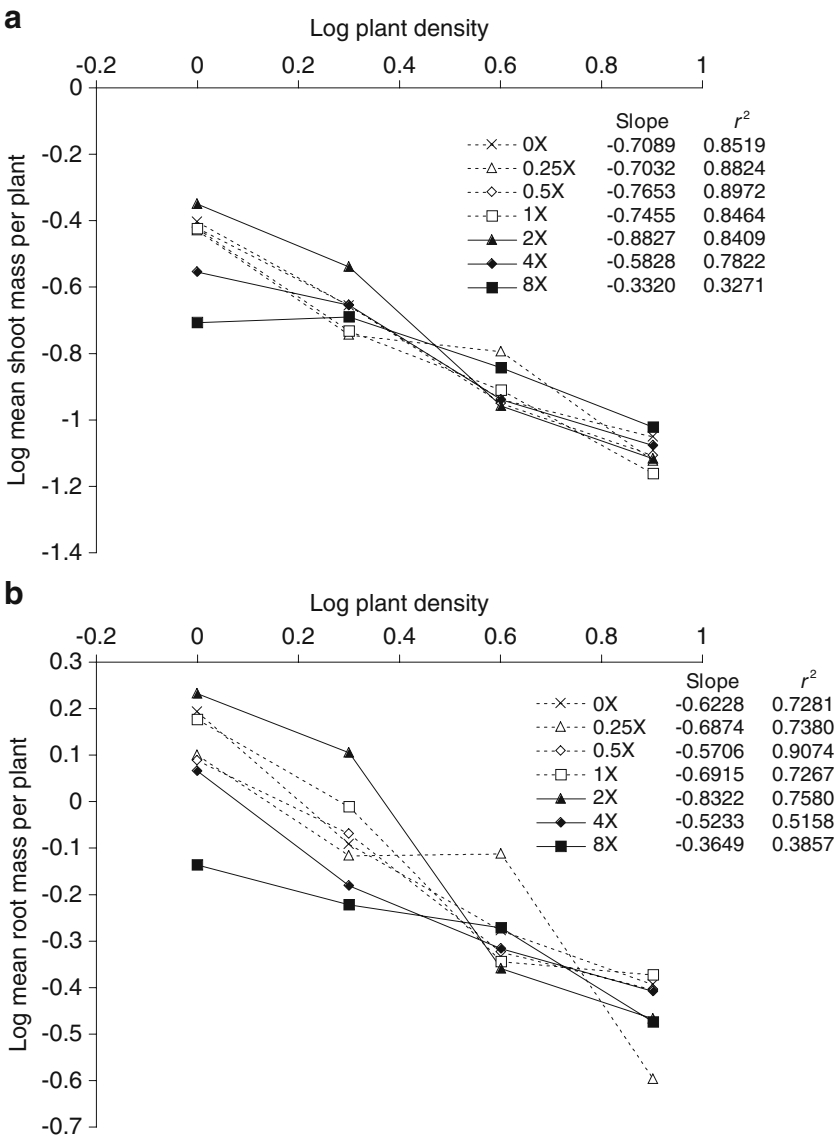

Fig. 4 Response of *I. pallida* plants exposed to a combined *A. petiolata* extract (N=2–3). **a** Relationship of log mean shoot mass per plant and log plant density. **b** Relationship of log mean root mass per plant and log plant density

impacting mean shoot and root dry masses (shoot: t=−15.34, P<0.001; root: t=−10.91, P<0.001; Fig. 4, Table 3). Extract dose significantly affected both mean shoot and root dry masses (shoot: t=−3.76, P<0.001; root: t=−3.35, P=0.001), with the strongest dose being the most inhibitory, but differences were significant only at the lowest density. Slopes of the regression lines for mean shoot mass and mean root mass against plant density were different (shoot: t=3.53, P<0.001; root: t=2.03, P=0.045) when pots received a combined extract (Fig. 4a). The highest dose led to the lowest slope, showing that at high doses allelopathic inhibition by *A. petiolata* is more important than resource competition between *I. pallida* plants. However, at the 1*X* dose expected to represent exposure levels in the field, the slope of the treatment regression lines could not be distinguished from that of control plants. Therefore, even though compounds produced by *A. petiolata* can inhibit *I. pallida* shoot and root growth, they do not appear to do so at an ecologically relevant dose.

The root-to-shoot ratios of plants exposed to a flavonoid-enriched fraction of an *A. petiolata* extract were affected by both density and dose (density: t=2.97, P=0.004; dose: t=2.16, P=0.034; Table 4). Root-to-shoot ratios were highest at a density of four plants per pot, indicating that, as *I. pallida* plants became more crowded, they invested resources preferentially in root growth, but these allocation patterns were not affected by exposure dose.

In *I. pallida* plants exposed to a glucosinolate-enriched fraction of an *A. petiolata* extract, root-to-shoot ratios generally were higher in plants exposed to low doses than

Table 3 Shoot and root dry masses of *I. pallida* plants exposed to a combined *A. petiolata* extract

Dose	One plant per pot	Two plants per pot	Four plants per pot	Eight plants per pot
Mean shoot dry mass per plant (mg±1 SE)[a]				
0*X*	394±93 ab	221±11 bcd	115±8 fghi	89±5 hi
0.25*X*	371±50 ab	180±6 cdef	161±25 cdefg	75±2 i
0.5*X*	378±62 ab	221±44 bcde	112±10 fghi	78±3 hi
1*X*	377±130 ab	185±18 cdef	122±6 efghi	69±2 i
2*X*	446±33 a	289±109 bc	110±7 fghi	76±4 i
4*X*	279±76 abc	221±16 bcd	115±11 fghi	84±3 hi
8*X*	196±48 cdef	204±71 cdef	144±33 defgh	95±12 ghi
Mean root dry mass per plant (mg±1 SE)[a]				
0*X*	1,557±320 ab	808±188 bcdefg	527±90 efghi	404±27 ghij
0.25*X*	1,257±180 abc	765±136 cdefgh	772±127 bcdefg	253±16 j
0.5*X*	1,224±122 abc	852±95 bcdef	474±11 efghij	394±24 ghij
1*X*	1,500±77 ab	974±197 abcde	452±88 fghij	424±106 ghij
2*X*	1,707±142 a	1,273±454 abc	437±40 fghij	341±19 ij
4*X*	1,162±291 abcd	659±127 cdefghi	483±126 fghij	390±96 hij
8*X*	730±108 cdefgh	600±194 defghi	535±126 efghij	336±77 ij

In dose column, *X* represents expected field levels of 3.3 mg *A. petiolata* tissue equivalents per gram soil

[a] Means with the same letter are not significantly different at α= 0.05 using Duncan's multiple-range test

in plants exposed to high doses (t=−2.54, P=0.013), showing that at high exposure levels root growth was slower than shoot growth (Table 5).

Since combined *A. petiolata* extracts affected both shoot and root dry mass of *I. pallida* plants similarly, root-to-shoot ratios were not affected by density or dose (density: t=1.62, P=0.108; dose: t=−0.33, P=0.743; Table 6).

Discussion

A. petiolata has the potential to exert strong allelopathic effects on surrounding plants, as shown by the reduced *I. pallida* growth observed in this study after exposure to high doses of either a flavonoid-enriched fraction or a combined extract. However, in contrast to reports of inhibition by glucosinolates at doses as low as two times field levels (Vaughn and Berhow 1999), we found no inhibition due to glucosinolates, even using a dose eight times higher than expected field levels. Studies that use multiple fractions are rare, but our finding that a combined extract had greater biological effects than each of its component parts supports previous work (Callaway et al. 2008).

Despite the well-known potential for *A. petiolata* to be allelopathic (Vaughn and Berhow 1999; Roberts and Anderson 2001; Prati and Bossdorf 2004; Stinson et al. 2006; Callaway et al. 2008), intraspecific resource competition was the dominant process determining growth of the *I. pallida* plants used in this study. Levels of glucosinolates and flavonoid glycosides in soils beneath *A. petiolata* plants in the field are low and often undetectable (Barto and Cipollini 2009). Furthermore, the half-lives of the glucosinolates and their degradates are frequently less than 10 days in nonsterile field soil (Gimsing et al. 2006, 2007), although, in nonsterile soil water, sinigrin has a half-life greater than 120 days (Tsao et al. 2000). The flavonoid glycosides are much less stable with half-lives less than 12 h in nonsterile

Table 4 Root-to-shoot ratios of dry masses of *I. pallida* plants exposed to a flavonoid-enriched fraction of an *A. petiolata* extract

Dose	(mean±1 SE)[a]			
	One plant per pot	Two plants per pot	Four plants per pot	Eight plants per pot
0*X*	4.05±0.23 ab	3.61±0.67 abc	4.51±0.52 ab	4.60±0.54 ab
0.25*X*	2.86±0.36 bc	3.71±0.71 abc	4.55±0.79 ab	4.69±0.68 ab
0.5*X*	3.52±0.60 abc	2.96±0.29 bc	4.28±0.13 ab	3.54±0.49 abc
1*X*	2.97±0.55 bc	4.03±0.38 ab	4.94±1.31 ab	4.18±0.36 ab
2*X*	2.25±0.34 c	3.41±0.18 abc	5.36±0.58 a	3.53±0.73 abc
4*X*	4.22±0.65 ab	3.92±0.53 ab	4.78±0.35 ab	3.67±0.99 abc
8*X*	5.05±1.13 ab	3.44±0.23 abc	4.59±0.48 ab	4.77±0.40 ab

[a] Means with the same letter are not significantly different at α=0.05 using Duncan's multiple-range test

Table 5 Root-to-shoot ratios of dry masses of *I. pallida* plants exposed to a glucosinolate-enriched fraction of an *A. petiolata* extract

Dose	(mean±1 SE)[a]			
	One plant per pot	Two plants per pot	Four plants per pot	Eight plants per pot
0*X*	4.05±0.23 ab	3.61±0.67 ab	4.51±0.52 ab	4.60±0.54 ab
0.25*X*	5.39±1.25 a	4.58±0.79 ab	3.90±0.23 ab	4.33±0.27 ab
0.5*X*	5.20±0.60 a	4.65±0.45 ab	4.16±0.96 ab	4.10±0.90 ab
1*X*	2.62±0.49 b	3.13±0.76 ab	4.52±1.00 ab	4.76±0.38 ab
2*X*	3.99±1.14 ab	5.31±1.12 a	3.86±0.36 ab	2.93±0.34 ab
4*X*	3.81±0.80 ab	3.68±0.58 ab	2.95±0.51 ab	3.65±0.58 ab
8*X*	2.63±0.34 b	3.28±0.27 ab	4.01±0.72 ab	3.98±0.34 ab

[a] Means with the same letter are not significantly different at α= 0.05 using Duncan's multiple-range test

field soil (Barto and Cipollini 2009). We did not assay the hexane fraction with lipophilic compounds, which may have contained more stable allelochemicals, although none have been reported for *A. petiolata*. We chose to use extracts instead of dried plant material so that our results would not be confounded by nutrient differences among the treatments. However, some allelochemicals may not have been extracted by our protocol and therefore would not have been applied to *I. pallida* plants. With these caveats, we can say that allelopathic effects due to the most abundant allelochemicals produced by *A. petiolata* are unlikely to be the most important factor contributing to its ability to displace *Impatiens* populations (McCarthy 1997). The 1*X* dose we used to represent expected field levels may be lower than actual field levels in sites with high *A. petiolata* densities or sites where degradation of allelochemicals is slowed. In such sites, allelopathy may still contribute to the invasive success of *A. petiolata*.

Invasive success may be explained by superior resource competition; however, seedlings of *I. capensis*, a smaller congeneric of *I. pallida*, were actually no less competitive than *A. petiolata* seedlings when both were planted at the same time (Meekins and McCarthy 1999). Although small *A. petiolata* seedlings did not outcompete *I. capensis*, *A. petiolata* is a biennial and bolts early in the spring of its second year before many other plants have grown substantially. Therefore, second-year *A. petiolata* plants likely are more competitive with an annual like *Impatiens* than *A. petiolata* seedlings and may be able to outcompete *I. pallida* seedlings. Superior resource competition was evident in a field study of second-year *A. petiolata* plants competing with *I. capensis*, but a portion of the competitive effects in that study were ameliorated by activated carbon (Cipollini et al. 2008b). Although we did not detect allelopathic effects on *I. pallida* at ecologically relevant doses, such effects may be more pronounced on target species that are less effective at competing for resources than *I. pallida*.

Further complicating matters are the interactions between direct (allelopathic) and indirect (resource competition) forms of competition. Resource competition can cause shifts in root-to-shoot ratios, which could alter levels of allelochemicals produced preferentially in roots or shoots (Goldberg 1990). Flavonoid glycosides are more abundant in the shoots of *A. petiolata*, while glucosinolates are more abundant in the roots (Vaughn and Berhow 1999). Other environmental factors such as soil texture, the presence of other soil contaminants, altitude, latitude, light levels, temperature, and pH of the soil also influence the production of allelochemicals (Blanco 2007). In *A. petiolata*, production of flavonoid glycosides varies seasonally (Haribal and Renwick 2001), and differences among populations may be due to environmental factors (Haribal and Renwick 2001) and/or be genetically controlled (Cipollini et al. 2005). Glucosinolate production also varies seasonally (Vaughn

Table 6 Root-to-shoot ratios of dry masses of *I. pallida* plants exposed to a combined *A. petiolata* extract

Dose	(mean±1 SE)[a]			
	One plant per pot	Two plants per pot	Four plants per pot	Eight plants per pot
0*X*	4.05±0.23	3.61±0.67	4.51±0.52	4.60±0.54
0.25*X*	3.47±0.52	4.24±0.75	4.98±0.98	3.38±0.32
0.5*X*	3.31±0.23	4.21±0.96	4.28±0.30	5.06±0.36
1*X*	4.75±1.13	5.56±1.69	3.63±0.56	6.21±1.66
2*X*	3.82±0.03	4.64±1.28	3.96±0.18	4.50±0.41
4*X*	4.39±1.00	2.93±0.35	4.15±1.02	4.75±1.29
8*X*	4.27±1.16	3.18±0.72	3.78±0.64	3.45±0.47

[a] There were no significant differences among means using Duncan's multiple-range test (α=0.05)

and Berhow 1999) and is influenced by environmental factors (Cipollini 2002). There has been a historical division in the literature between advocates of allelopathy and resource competition, leading many to attempt to prove that either one or the other is dominant. In reality, both are facets of the same phenomena, competition, and attempts to incorporate both may prove more fruitful than focusing on either one independently.

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ERRATUM

Vicia faba-Lygus rugulipennis Interactions: Induced Plant Volatiles and Sex Pheromone Enhancement

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The presentation of Table 1 in the original version of this article unfortunately contained a mistake. The corrected table is given below. Springer regrets the error.

Table 1 Mean amount±SE of volatiles collected from healthy *Vicia faba* plants, plants damaged by *Lygus rugulipennis* males that had been removed, plants damaged by females that had been removed, plants damaged by males still present, plants damaged by females still present

Treatment	*N*	Compounds (ng/h)[a]					
		Alcohols		Alkaloids Indole	Esters		
		(Z)-3-Hexenol	1-Hexanol		(Z)-3-hexenyl acetate	Hexyl acetate	Methyl salicylate
Healthy	7	194.86±40.87a	2.69±0.70a	1.82±0.44a	55.28±16.42a	0.36±0.20b	0.44±0.04c
Damaged by ♂	7	224.07±65.14a	2.37±0.57a	2.29±0.58a	47.13±10.04a	0.09±0.06b	2.00±0.54bc
Damaged by ♀	7	196.80±48.34a	1.96±0.41a	2.27±0.48a	52.11±9.94a	0.20±0.15ab	2.86±0.65ab
Damaged+♂	7	193.29±34.98a	2.04±0.33a	3.51±1.33a	41.47±9.90a	0.42±0.24ab	2.40±0.58b
Damaged+♀	7	94.90±34.21a	1.82±0.50a	2.95±1.13a	81.55±46.48a	1.41±0.41a	8.90±2.98a

Total duration of volatile collection was 24 h, starting at ~11.00–12.00 am

[a] The mean amount of each compound (ng/h) was estimated by comparison of peak area with that of an internal standard (dodecane, 100 ng). Numbers in column followed by the same letter indicate means that are not significantly different ($P>0.05$, ANOVA).

N number of replicates, TMTT (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene

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Defense by Volatiles in Leaf-Mining Insect Larvae

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Abstract The defense strategy of an insect toward natural enemies can include a trait that appears at first sight to contradict its defensive function. We explored phylogeny, chemistry, and defense efficiency of a peculiar group of hymenopteran sawfly larvae where this contradiction is obvious. Pseudodineurini larvae live in leaf mines that protect them from some enemies. Disturbed larvae also emit a clearly perceptible lemon-like odor produced by ventral glands, although the mine hampers the evaporation of the secretion. The mine could also lead to autointoxication of a larva by its own emitted volatiles. Citral was the major component in all Pseudodineurini species, and it efficiently repels ants. We conclude that full-grown larvae that leave their mine to pupate in the soil benefit from citral by avoiding attacks from ground-dwelling arthropods such as ants. In some species, we also detected biosynthetically related compounds, two 8-oxocitral diastereomers (i.e., (2*E*,6*E*)- and (2*E*,6*Z*)-2,6-dimethylocta-2,6-dienedial). Synthetic 8-oxocitral proved to be a potent fungicide, but not an ant repellent. The discrete distribution of 8-oxocitral was unrelated to species grouping in the phylogenetic tree. In contrast, we discovered that its presence was associated with species from humid and cold zones but absent in species favoring warm and dry environments. The former should be protected by 8-oxocitral when faced with a fungal infestation while crawling into the soil. Our work shows the importance of integrating knowledge about behavior, morphology, and life history stages for understanding the complex evolution of insects and especially their defense strategies.

Keywords Sawfly larvae · Nematinae · Ventral glands · *Pseudodineura* · Leaf-mining insect · Monoterpenes · Citral · 8-Oxocitral · Ant repellent and antifungal activities · Abiotic factors

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Introduction

Predation pressure is a potent evolutionary force especially on insects, which are a basic element in most terrestrial ecosystems. As a consequence, defense strategies are widespread and diversified in insects and often rely on the use of noxious chemicals (Eisner 1970; Blum 1981). Chemical defense mechanisms have a profound impact on the entire biology of an insect species, as defense is coupled with other adaptations in morphology, physiology, niche use, behavior, etc. (Whitman et al. 1990). Other nonchemical defensive traits also interact in the evolution of insect species (Evans and Schmidt 1990). A trait in isolation may appear at first glance to be in contradiction to the others. For instance, conspicuousness renders insects more visible,

thus, more vulnerable; but this trait is used in defense strategies such as aposematism and mimicry, by relying on the predator's associative learning capabilities (Guilford 1990; Ruxton and Sherratt 2006).

We detected such a paradoxical situation in the biology of a group of phytophagous insects that not only live within a plant tissue but also emit defensive volatiles. The tribe Pseudodineurini belongs to the hymenopteran nematine (Tenthredinidae) sawflies (see Boevé (2008) for an introduction to the family). The larvae of this tribe are specialized leaf miners on plants mainly belonging to the buttercup family Ranunculaceae (Altenhofer 2003; Altenhofer and Pschorn-Walcher 2006; Table 1, Fig. 1). Compared to free-living sawfly larvae, endophytic larvae presumably are mechanically protected from some natural enemies (Price and Pschorn-Walcher 1988; Connor and Taverner 1997). Additionally, almost all larvae of the 1,200 highly diverse nematine species possess ventral glands that emit a volatile antipredator secretion (Boevé and Pasteels 1985). A typical lemon-like odor is perceived easily when Pseudodineurini larvae are disturbed. The odor intrigued us since volatiles often repel (at a distance) approaching and/or attacking small arthropods (Pasteels et al. 1983). For example, the odor of lemon eucalyptus repels insects such as mosquitoes (Moore et al. 2007). Further, several nematine species among the free-living *Nematus* and galling *Pontania* show a secondary reduction in gland size (Boevé and Pasteels 1985), no perceptible odor, only a trace of volatiles such as long chain hydrocarbons (Boevé et al. 1992), and thus probably a more or less functionless secretion. Pseudodinerini larvae are endophytic and emit repellents, and our aim was to understand this apparent redundancy of defensive traits. The latter trait is especially puzzling since the closed environment in which the larva lives hampers the evaporation of volatiles and could lead to autointoxication.

Pseudodineurini is a small tribe that comprises about 15 described Holarctic species (Taeger and Blank 2008). A dozen belong to the genus *Pseudodineura*, and larvae are known for seven Western Palaearctic species. Adults are about 5 mm in body length, and larvae up to 1 cm. Several species of the tribe are restricted locally and occur only during a short season (Altenhofer and Pschorn-Walcher 2006). However, we were able to gather enough material for chemical and phylogenetic analyses. Preliminary chemical analyses revealed the occurrence of two acyclic monoterpene diastereomers (*E*)-3,7-dimethyl-2,6-octadienal (geranial) and (*Z*)-3,7-dimethyl-2,6-octadienal (neral; the mixture of the two compounds is called citral) in the glandular secretion of *Pseudodineura* larvae. These compounds also occur in lemon, lemon grass, etc. and are important in the food and perfume industry. Their occurrence was expected in *Pseudodineura* due to the perceptible odor and because free-living *Nematinus* larvae also emit them (Boevé et al. 1984). However, two isomers of another unidentified monoterpene occurred in only some *Pseudodineura* species. In the present study, the chemical profile of the glandular secretion of this group was investigated in relation to phylogeny. Moreover, bioassays were performed to reveal the defensive efficiency of live larvae and to compare the bioactivity of citral and the other monoterpenes. The results lead us to an explanatory framework that brings together the evolution and ecology that includes biotic and abiotic factors. As a case study, it allows us to better comprehend how multifunctional traits can evolve.

Methods and Material

Sample Collection Sawflies were collected in the field, mainly in the province *Niederösterreich* of Austria (Table 1). The Pseudodineurini larvae were identified by EA, and voucher specimens of all sawfly samples are kept by JLB at the Royal Belgian Institute of Natural Sciences.

Phylogenetic Analyses Twenty-five specimens representing all West Palearctic Pseudodineurini species except *Pseudodineura heringi* were used for phylogenetic analyses. We also added one North American species (*Pseudodineura parva*). Six outgroup species were chosen on the basis of the subfamilial phylogeny of Nematinae proposed by Nyman et al. (2006).

Total genomic DNA was extracted by using DNeasy Mini Kits (Qiagen). The posterior half of the larvae (or the whole specimen when larvae were smaller than 5 mm) stored in 96% ethanol was used. Fragments of the mitochondrial cytochrome *b* gene (cob; aligned length: 433 bp) were amplified and sequenced by using the primers CB-J-10933: 5′-TATGTACTACCATGAGGACAAATATC-3′, and CB-N-11367: 5′-ATTACACCTCCTAATTTATTAGG AAT-3′ (Simon et al. 1994). The cytochrome *c* oxidase subunit I gene (COI; aligned length: 874 bp) was amplified and sequenced with the primers sym-C1-J-1718 (Nyman et al. 2006) and A2590 (Normark et al. 1999). A fragment of the nuclear 28S rRNA gene (28S; aligned length: 579 bp) was amplified and sequenced with the primers D2F: 5′-CGTGTTGCTTGATAGTGCAGC-3′ and D2R: 5′-TTG GTCCGTGTTTCAAGACGG-3′ (Schmidt et al. 2006). PCR products were sequenced on an ABI Prism 3130XL capillary sequencer. For detailed list of samples and GenBank accession numbers, see Table 1. DNA sequences of 28S rRNA were aligned with MAFFT 6 (Katoh et al. 2002; Katoh and Toh 2008) by using the Q-INS-i option. Mitochondrial gene sequences were aligned manually.

Phylogenetic analyses were carried out with PAUP* v4.0b10 (Swofford 2002), MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003), and BEAST v1.4.8. (Drummond and

Rambaut 2007). Trees were calculated with parsimony (gaps in 28S sequences were considered as fifth character), maximum likelihood (ML), and Bayesian (BI) methods based on each gene separately, on the concatenated sequences of two genes (COI and 28S), and on the concatenated sequences of three genes. The Akaike and the Bayesian information criteria implemented in jModeltest v0.1.1 (Posada 2008) were used to find appropriate nucleotide substitution models, and the recommended settings were used in the subsequent ML and BI analyses. In order to infer branch supports, we performed 2,000 bootstrap replicates under parsimony and 100 replicates under ML criteria using PAUP*. BI analyses were carried out with MrBayes running 2×10^6 generations. For the protein-coding genes (i.e., COI as in Fig. 2), we partitioned the dataset according to codon positions (first and second positions vs. third positions), and performed a Bayesian analysis in BEAST running 10^7 generations. In all Bayesian analyses, the first 25% of the sampled trees were discarded ("burn-in").

Chemical Analyses and Syntheses Full-grown Pseudodineurini larvae were taken out of their leaf mines, and samples containing 13 to 30 individuals were extracted for 5 min in approximately 0.5 ml hexane. For *Pseudodineura fuscula*, larvae as well as eonymphs were extracted in this way. Extracts were kept at −80°C until gas chromatography/mass spectroscopy (GC–MS) analysis.

Most analyses were performed on a Fisons 8060 GC system equipped with a nonpolar capillary column (DB-5, 30 m×0.32 mm I.D., film thickness 0.25 μm) and coupled to a Fisons MD800 quadrupole mass spectrometer. The extract was concentrated, up to 0.25 ml, when relatively few larvae were extracted. From each extract, 1 μl was injected, with a solvent delay of 3 min. The temperature program was 2 min at 60°C, then up to 280°C with 3°C min^{-1}, and then 30 min at 280°C. Spectra were obtained in EI mode.

To confirm the chemical composition of some extracts and to elucidate the structure of a monoterpene that remained unidentified, additional analyses were performed on a Hewlett Packard GC 6890 with split/splitless-injector and a fused silica capillary column (BPX5, SGE Inc., 25 m×0.22 mm I.D., film thickness: 0.25 μm) connected to a Hewlett Packard MSD 5973 quadrupole mass spectrometer operating in the EI mode. The temperature program was 1 min at 50°C, then up to 320°C with 10°C min^{-1}.

Neral (**c1**) and geranial (**c2**) were synthesized from nerol and geraniol via Swern oxidation (Omura and Swern 1978). The obtained **c1** and **c2** contained 1.4% of **c2** and 1.1% of **c1**, respectively. The 8-oxocitrals also were synthesized from nerol and geraniol. First, an allylic oxidation was performed by using catalytic amounts of selenium dioxide and tert-butyl hydroperoxide as cooxidant (Li et al. 1997). The resulting dialcohols then were oxidized via Swern oxidation (Omura and Swern 1978). (2*E*,6*Z*)-2,6-Dimethylocta-2,6-dienedial (**c3**) contained 25% of (2*E*,6*E*)-2,6-dimethylocta-2,6-dienedial (**c4**), and **c4** contained 10% of **c3**. As far as we know, no selective synthesis exists that would furnish these compounds in the same diastereomeric purity as obtained for the citrals. The 8-oxocitrals are relatively unstable, and they slowly isomerize upon standing. Only the C-6 double bond isomerizes, thus, showing the higher reactivity of 8-oxocitral at this position (Chan et al. 1968). Before performing the bioassays (see below), the relative proportion of the mixtures **c3** + **c4** had already changed from 3:1 to 1:1 and from 1:9 to 1:4. After these bioassays, which required several months, the mixtures were reanalyzed, and their relative proportions remained stable for the mixture 1:1, whereas the other mixture was largely degraded.

Bioassays Three types of bioassays were performed: sawfly larvae alive vs. ants, synthesized compounds vs. ants, and these same compounds vs. fungi.

The interactions between larvae of *Pseudodineura mentiens* and ant workers of *Myrmica rubra*—a common ant species in Europe, including Austria (Seifert 1988)—were observed in 9-cm diam Petri dishes. First, one leaf of *Hepatica nobilis* with a full-grown larva still in its mine was deposited in a Petri dish containing ten ants. Six replications were made. After 24 h, we observed whether the ants opened the mine. Second, ten ants per Petri dish were confronted with one larva just taken out of its mine. Seven full-grown and six penultimate instar larvae were tested. We counted after 15 min the number of ants that surrounded the larva, i.e., clearly making a mandibular contact with it. After the bioassay, we determined the instar by recording the head coloration and measuring the head capsule width (HCW). Full-grown larvae have a whitish head and HCW=1.0 to 1.18 mm, whereas larvae at penultimate instar have a dark head and HCW=0.85 to 1.0 mm.

To measure the repellent effect of the synthesized monoterpene isomers (see above) we used the ant *Crematogaster scutellaris* in an experimental setup described in Boevé (1988) and in which this ant species performed better than *M. rubra*. Forty ant workers were taken from a laboratory colony and left for 30 min in a 14-cm diam Petri dish coated on its inner border with Fluon® to prevent escape. Then, a 5×5-cm glass plate with a metallic podium of 1 cm diam and 0.5 cm in height fixed at its center was placed in the uncovered Petri dish. The plate had 75 μl of a 1:1 water/honey solution deposited on the glass around the podium. For 5 min, ants were allowed to find the solution and feed. The number of feeding ants then was counted for

Table 1 Sawfly samples used in chemical (GC-MS) and genetic (DNA) analyses

Taxon	Field host-plant	Voucher, stage[a]	Locality[b]	Date	Collector[c]	GC-MS[d]	DNA[e]	COI[f]	Cob[f]	28S[f]
Pseudodineura clematidis (Hering)	*Clematis alpina*	P1625, L	Umballfälle	08.06.1999	EA	+				
		P2252, L	Umballfälle	31.07.2002	EA		.1	FJ858810	FJ858866	FJ858842
							.2	FJ858811	FJ858867	FJ858843
P. clematidisrectae Hering	*Clematis recta*	P1584, L	Langenlois	20.05.1998	EA	+				
		P2169, L	Langenlois	25.05.2002	EA		.1	FJ858809	FJ858861	FJ858837
							.2	FJ858815	FJ858862	FJ858838
							.3	FJ858820	FJ858863	FJ858839
		P2901, L	Langenlois	04.06.2008	EA		.1	–	FJ858873	FJ858852
							.2	FJ858828	FJ858874	FJ858853
							.3	–	FJ858875	FJ858854
P. enslini (Hering)	*Trollius europaeus*	P1588, L	Allentsteig	02.06.1998	EA	+				
		P2190, L	Ötscher	05.07.2002	EA		.1	FJ858817	FJ858865	FJ858841
		P2900, L	Allentsteig	04.06.2008	EA		.1	FJ858825	–	FJ858849
							.2	FJ858826	–	FJ858850
							.3	FJ858827	–	FJ858851
P. fuscula (Klug)	*Ranunculus* spp.	P1587, L on *R.b.*	Hernstein	01.06.1998	EA	+				
		P1632, L on *R.p.*	Arbesbach	13.06.1999	EA	+				
		P2167, L on *R.p.*	Groβ Gerungs	09.06.2002	EA		.1	FJ858812	FJ858859	FJ858835
							.2	–	–	–
							.3	FJ858819	FJ858860	FJ858836
		P2899, L on *R.p.*	Groβ Gerungs	04.06.2008	EA		.1	FJ858822	FJ858870	FJ858846
							.2	FJ858823	FJ858871	FJ858847
							.3	FJ858824	FJ858872	FJ858848
		P2848, A	Nana Aseme (E)	21.04.2002	MH		.1	FJ858818	FJ858868	FJ858844
P. heringi (Enslin)	*Anemone sylvestris*	P1589, L	Langenlois	01.06.1998	EA	+				
P. mentiens (Thomson)	*Hepatica nobilis*	P1635, L	Neulengbach	29.06.1999	EA	+				
		P2842, L	Langenlois	24.08.2008	EA	+				
		P2172, L	Neulengbach	03.07.2002	EA		.1	FJ858816	FJ858864	FJ858840
P. parvula (Klug)	*Pulsatilla* spp.	P1590, L on *P.v.*	Langenlois	01.06.1998	EA	+				
		P2168, L on *P.p.*	Langenlois	25.05.2002	EA		.1	FJ858813	–	–
							.2	FJ858814	–	–
P. parva (Norton)	(*Hepatica*)	P2849, A	Petersham (M)	05.2002	TN		.1	FJ858821	FJ858869	FJ858845
Endophytus anemones (Hering)	*Anemone nemorosa*	P2573, L	Etzen	08.05.2005	EA	+				
		P2098, L	Etzen	05.05.2002			.1	FJ858808	–	FJ858834
Dineura pullior Schmidt & Walter	*Betula*	P1809, Eo	lab rearing (F)	04.2001	AK		.1	FJ858807	–	FJ858831

Hemichroa crocea (Geoffroy)	*Alnus glutinosa*	P1775, L	Grimminge (B)	04.09.2000	JLB		.1	FJ858804	FJ858856	FJ858830
Nematinus fuscipennis (Serville)	(*Alnus*)	P2078, A	Illfeld (G)	20.05.2001	JLB		.1	FJ858806	FJ858858	FJ858833
Stauronematus compressicornis (Fabricius)	*Populus tremula*	P1752, L	Wellin (B)	01.08.2000	JLB		.1	FJ858803	FJ858855	FJ858829
Cladius pectinicornis (Geoffroy)	*Rosa* sp.	P2072, L	Delémont (S)	21.08.2000	US		.1	FJ858805	FJ858857	FJ858832

L larva, *Eo* eonymph, *A* adult, *R.b. Ranunculus bulbosus*, *R.p. R. platanifolium*, *P.v. Pulsatilla vulgaris*, *P.p. P. pratensis*, *E* Estonia, *M* Massachusetts, *F* Finland, *B* Belgium, *G* Germany, *S* Switzerland, *MH* Mike Heidemaa, *TN* Tommi Nyman, *AK* Antti Kause, *US* Urs Schaffner

[a] The corresponding larval host-plant given between (parentheses), and, when necessary, the plant species

[b] If not Austria, the country of the locality is mentioned

[c] Collectors, if not the authors, are MH, TN, AK, and US

[d] The Pseudodineurini larvae where always collected, per sample, in a small area, thus, clearly belonging to a single population. Their host-plants occurred as a small patch, especially *Clematis recta*, *Ranunculus platanifolium* from Groß Gerungs, and *Pulsatilla vulgaris*. Larvae used in chemical analyses are marked (+)

[e] For genetic analyses, we often used more than one individual from one sawfly population, each one being numbered (.1, .2, .3) as DNA individual. This specimen numbering is reused in Fig. 2

[f] GenBank accession numbers are given for COI, cob, and 28S; sequence could not be obtained (–)

Fig. 1 Sawfly larva of *Pseudodineura fuscula* on *Ranunculus platanifolium*. All *Pseudodineura* species are leaf miners of Ranunculaceae plants. Both leaf epidermis layers being translucent, the larva is visible as is the feces that accumulate in the mine. Photographed by J-L Boevé

the first time (t=0), and simultaneously, a piece of 5×5-mm filter paper embedded with 0.25 µl of the tested monoterpene was placed on the podium. The number of feeding ants was recounted, once per minute, up to t=10 min. This experiment, performed at 25°C, was replicated four times per monoterpene. Distilled water instead of a monoterpene was used as a control.

Antifungal testing of **c1**, **c2**, **c3** + **c4** (1:1 and 1:4) was performed with the yeast *Candida albicans* (strain: IHEM 9559) and the filamentous fungus *Aspergillus fumigatus* (IHEM 18963). These strains are referenced in the BCCM/IHEM Collection catalog (http://bccm.belspo.be/db/ihem_search_form.php). These two human pathogenic species were chosen because standardized reference methods are available for antifungal susceptibility testing (i.e., CLSI M27A (2002a) for yeasts and CLSI M38A (2002b) for filamentous fungi). These tests are performed in 96-well plates. We used an individual microplate per sample to avoid interferences due to the volatility of the compounds. Stock inoculum suspensions were prepared from a 24-h-old culture of *C. albicans* grown on Sabouraud medium, and from a 7-day-old culture of *A. fumigatus* grown on potato dextrose agar. As starting sample amounts, we used 3.75, 4.5, 5.0, and 3.0 mg of **c1**, **c2**, **c3** + **c4** (1:1), and **c3** + **c4** (1:4), respectively. The sample was solubilized in 200 µl of sterile saline. The microplate contained 100 µl of twofold serial dilutions of the sample and 100 µl of the fungal inoculum in twice concentrated RPMI medium (2% glucose, 0.165M MOPS). The final concentration of the inoculum was 2.5 10^3 CFU/ml for *C. albicans* and 5 10^3 CFU/ml for *A. fumigatus*. Growth and sterility controls were included in each experiment. Microplates were

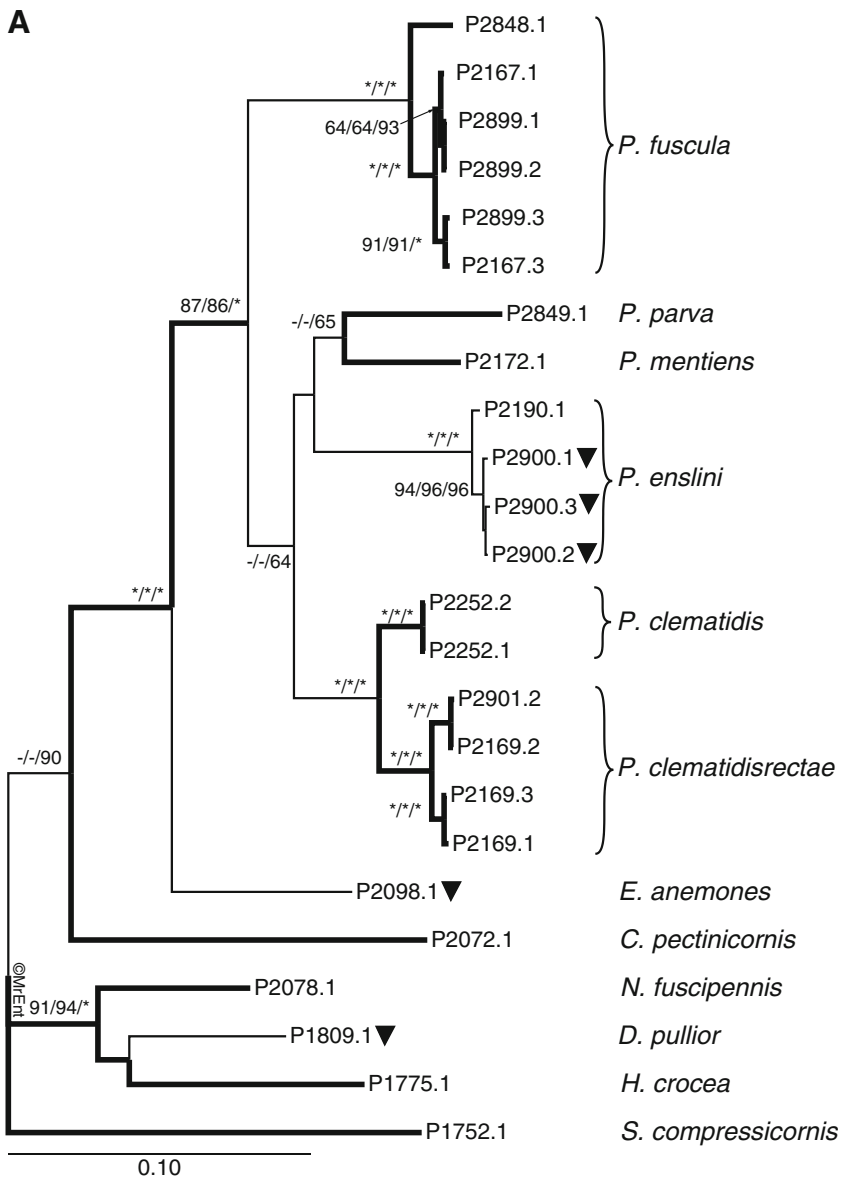


Fig. 2 Phylogenetic relationships of the Pseudodineurini (**A**) and *Pseudodineura* (**B**). In **A**, the tree was reconstructed using Bayesian inference for concatenated COI and 28S sequences (1,453 bp). Branches in *bold* show clades supported by the combined analyses of COI, cob, and 28 S sequences (1,886 bp). Taxa marked with *black inverted triangles* were not included in the latter analysis. Clade support values are: parsimony bootstraps (2,000 replicates)/maximum likelihood bootstraps (100 replicates)/ Bayesian posterior probabilities (2×10^6 generations). Values under 60% are not shown. Bootstrap values of 97–100% and posterior probabilities of 100% are replaced by *asterisks*. In **B**, the tree from COI sequences was reconstructed using BEAST, and posterior probabilities were calculated by running 10^7 generations. *Asterisks* replace values between 99% and 100%. Presence (*plus sign*) or absence (*minus sign*) of 8-oxocitral (**c3** and **c4**). Species documented as xerotherm (*Yes*) or not (*No*) in Altenhofer and Pschorn-Walcher (2006). Unknown or unclear (*question mark*)

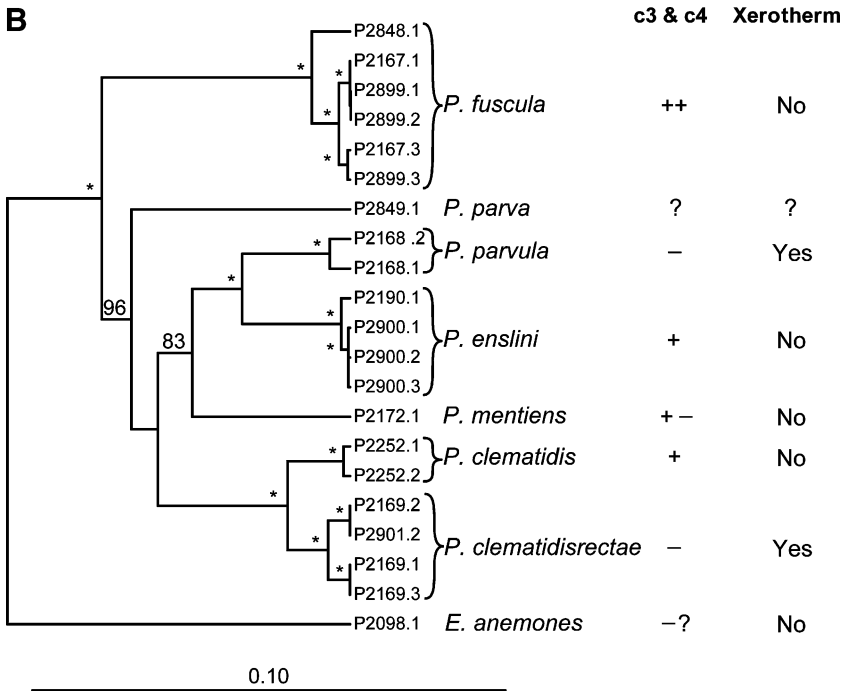
incubated at 35°C for 48 h and analyzed by spectrophotometry at 405 nm. The minimum inhibitory concentration (MIC)100 corresponds to a total growth inhibition, and MIC50 to a 50% growth inhibition. The minimum fungicidal activity (MFC) is the lethal concentration determined by inoculation on an agar plate of 20 µl aliquots from each well and that showed 100% growth inhibition compared to the growth control. Thus, the MFC is the lowest concentration resulting in no growth.

Results

Phylogenetic Analyses Complete datasets of COI, cob, and 28S sequences (total aligned length: 1866 bp) were obtained for representatives of ten species. For *Pseudodineura parvula*, only the amplification and sequencing of COI was successful; for *Endophytus anemones* and *Dineura pullior* we obtained COI and 28S sequences.

All combined and single-gene analyses (except 28S) supported monophyly of the genus *Pseudodineura* with highest bootstrap and posterior probability values (Fig. 2A). *Endophytus anemones* appeared to be the sister taxon of *Pseudodineura*. Phylogenetic relationships among *Pseudodineura* species could not be completely resolved despite the different evolutionary characteristics and amount of phylogenetic information of the three selected markers. Nonetheless, the sister-species relationship of *Pseudodineura clematidis* and *Pseudodineura clematidisrectae* consistently received highest support. Furthermore, COI analyses revealed a probable sister-species relationship between *Pseudodineura parvula* and *Pseudodineura enslini* (Fig. 2B).

Fig. 2 (continued)



Chemical Analyses The glandular secretion was composed mainly of monoterpenes (Fig. 3). Neral (**c1**) and geranial (**c2**) were the major compounds in all studied Pseudodineurini. In several *Pseudodineura* species, an uncommon monoterpene occurred as two isomers in medium amounts. The mass spectra showed a molecular mass 14 amu higher than that of citral, which is consistent with an additional methylene group or carbonyl function. The tabular data of the mass spectrum were comparable with those for (2*E*,6*E*)-2,6-dimethylocta-2,6-dienedial (**c4**) as described in literature (Veith et al. 1996). The structure then was confirmed by synthesis, followed by comparison of mass spectra and retention indices of the natural and synthetic compounds. The analogous (2*E*,6*Z*)-2,6-dimethylocta-2,6-dienedial (**c3**) was identified in the same way.

For each species, the relative abundance (in percentage) of **c1**, **c2**, **c3**, and **c4** was as follows (see also Fig. 2B): *P. clematidisrectae* (29, 53, 0, 0, respectively), *P. clematidis* (24, 67, 2, 3), *P. parvula* feeding on *Pulsatilla vulgaris* (34, 57, 0, 0), *P. enslini* (15, 63, 3, 10), *P. fuscula* feeding on *Ranunculus bulbosus* (35, 56, 1, 3), *P. fuscula* feeding on *Ranunculus platanifolium* (10, 55, 2, 13), *P. heringi* (31, 46, 0, 0), *P. mentiens* (25, 53, 1, 2 for a sample from June; 25, 32, 0, 0 for a sample from August; 25, 52, 0, 0 for eonymphs from this sample), and *E. anemones* (35, 65, 0, 0). Thus, the relative abundance varied between 10% and 37% for **c1**, 46–72% for **c2**, 1–3% for **c3**, and 3–13% for **c4**, which means that **c2** was always more abundant than **c1**, and **c4** more than **c3**.

Bioassays The six *P. mentiens* larvae left in their leaf mine survived well their 24 h confrontation with ant workers of *M. rubra*, since no mine was opened during this period. In the bioassay where larvae were experimentally taken from their mine, the mean number (± SD) of ants surrounding a single penultimate instar larva was 1.7 (± 0.8), but none surrounded a full-grown larva ($P<0.01$, Mann–Whitney U test).

The two isomers of citral (**c1** and **c2**) were significantly repellent for *C. scutellaris* ant workers, with compound **c1**

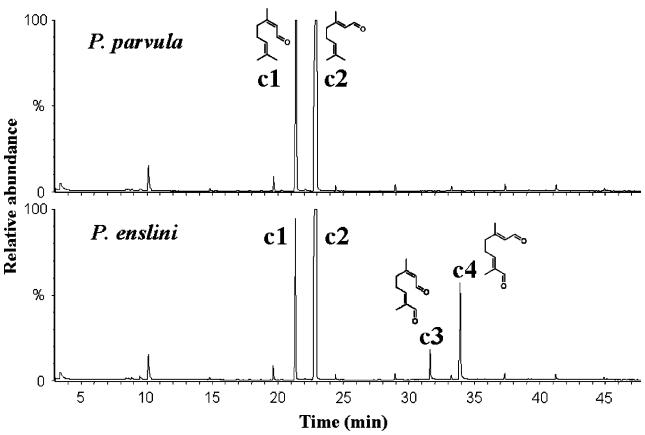


Fig. 3 Chromatograms of the volatiles emitted by two closely related *Pseudodineura* species, *P. parvula* and *P. enslini*. The monoterpene isomers correspond to citral (**c1** and **c2**) and 8-oxocitral (**c3** and **c4**). Other, smaller peaks correspond to alkanes: from dodecane with t_R= 10.13 to octadecane with t_R=44.95

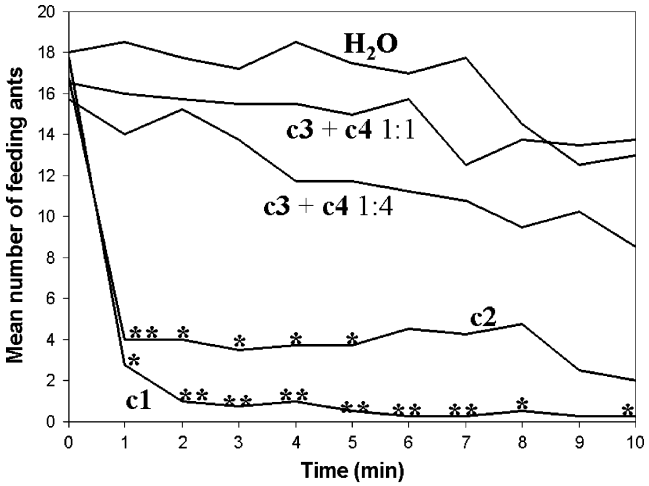


Fig. 4 Repellent activity of citral (**c1** and **c2**) and related 8-oxocitral (**c3** and **c4**) on the ant *C. scutellaris*. The monoterpenes were offered at $t=0$ on a small podium that was surrounded at its base by feeding ants. The number of feeding ants was counted every minute during 10 min. Water was used as a control. Standard deviation values are not shown, for clarity. $*P<0.05$, $**P<0.01$, Kruskall–Wallis test with post-test (corrected for ties), by comparing each volatile vs. water

being slightly more repellent than **c2**. In contrast, the mixture of the two isomers of 8-oxocitral (**c3** and **c4**) showed only a weak nonsignificant activity (Fig. 4).

The antifungal susceptibility tests revealed that all four synthesized monoterpenes were active against fungi (Table 2). The mixture **c3** + **c4** (1:1) showed the strongest fungicidal activity (with MFC=0.781 mg/ml), for both *C. albicans* and *A. fumigatus*, compared to the other tested monoterpenes. We consider activity values as similar between the two fungi species, taking into account that such values obtained from terpenes can vary even between strains of the same species (Sabini et al. 2006).

GC–MS analyses, performed after the bioassays, revealed that only the mixture **c3** + **c4** (1:1) remained stable over time, whereas **c3** + **c4** (1:4) was degraded. This may explain why a relatively low antifungal activity was observed with **c3** + **c4** (1:4), which is, however, closer to the natural mixture (see Fig. 3). It is possible that **c3** is more active than **c4**, which would increase the potency of **c3** + **c4** (1:1) mixture where **c3** is proportionally more concentrated.

Discussion

There are both advantages and disadvantages for an insect to feed as a leaf miner, as compared to an external leaf feeder (Connor and Taverner 1997). From the point of view of aggression from natural enemies, the risk of external disease infection is lower, whereas parasitoids inflict higher mortality due to a lower mobility of the leaf miner. Galling nematines may be less susceptible to this kind of parasitism than free-living species, since the mean number of parasitoid species is 4.0 for shoot-galling nematines, in contrast to almost 16 for free-living colonial nematines (Price and Pschorn-Walcher 1988; but see Nyman et al. 2007). Among the Pseudodineurini, 0–3 parasitoid species per host species were recorded (Altenhofer and Pschorn-Walcher 2006). Thus, it is likely that the leaf-mining habit allows Pseudodineurini larvae to avoid at least some parasitoids, such as tachinid flies. Considering predation, mining vs. external feeding habits seem to be similar in risks incurred (Connor and Taverner 1997). The mechanical protection offered by the mine was effective in our bioassay where larvae and mines remained intact for 24 h while surrounded by *M. rubra* workers. Mechanical protection appears to be insufficient, however, in the birch leaf mining sawfly *Fenusa pusilla*. It feeds in newly unfolding thin leaves and can be preyed upon by ants (Pezzolesi and Hager 1994). This sawfly species does not belong to the nematines and does not possess ventral glands. Thus, predation by ants may have a significant impact on sawfly leaf miners if the leaf epidermal layers are thin and if the larvae are not defended chemically.

Citral was the major compound in all analyzed Pseudodineurini species (Fig. 3), and it was detected in full-grown larvae still in their mine as well as in eonymphs in their

Table 2 In vitro susceptibility of two fungal species *Candida albicans* and *Aspergillus fumigatus* to citral (**c1** and **c2**) and mixtures of 8-oxocitrals (**c3** and **c4**)

Monoterpenes	Fungus	MIC50 (mg/ml)	MIC100 and MFC (mg/ml)[a]
c1	*C. albicans*	1.172	2.344
	A. fumigatus	2.343	4.687
c2	*C. albicans*	1.406	2.812
	A. fumigatus	5.625	11.25
c3 + **c4** (1:1)	*C. albicans*	0.39	0.781
	A. fumigatus	0.39	0.781
c3 + **c4** (1:4)	*C. albicans*	no MIC50	3.75
	A. fumigatus	0.937	1.875

[a] MFC and MIC100 values were identical in all tests

MIC50 minimum inhibitory concentration 50%, *MIC100* minimum inhibitory concentration 100%

cocoons. It is an insect repellent (Boevé 1988; Vartak et al. 1994), fungicide, and a fungus growth inhibitor (Kuwahara et al. 1989; Lima et al. 2005; Sabini et al. 2006). These bioactivities were corroborated by our bioassays (Fig. 4, Table 2). We believe that citral will be used especially when the larva leaves a mine. The larva will then crawl into the soil to spin a cocoon (Hering 1951; Zinovjev and Vikberg 1998; Altenhofer and Pschorn-Walcher 2006) and may be easily attacked by foraging ground-dwelling arthropods such as ants. A similar situation is found in another endophytic nematine, the apple sawfly *Hoplocampa testudinea*, which also pupates in the soil. The size of ventral glands increases in an allometric way once the larva becomes full-grown, the glandular secretion then becomes obvious, and the defense is efficient against ants (Boevé et al. 1997). The ontogenic increase in *P. mentiens* chemical defense was reflected in our bioassays by the significant difference between full-grown larvae, which were not approached, and younger ones.

There seems to be no direct influence of the host-plant on the biosynthesis of monoterpenes by Pseudodineurini larvae. Citral is the major compound of the glandular secretion not only in the Pseudodineurini but also in the alder-feeding *Nematinus* species (Boevé et al. 1984), and it is detected in two spruce-feeding *Pristiphora* species, *Pristiphora compressa* and *Pristiphora pallida* (Boevé, personal observation). The independence between host-plant (chemistry) and chemical composition of the glandular secretion also is observed in other nematine sawflies: for benzaldehyde produced by *Nematus* and *Pristiphora* species and for dolichodial produced by *Nematus* and *Craesus* species (Boevé et al. 1992; Boevé and Heilporn 2009). Thus, these genera as well as Pseudodineurini probably produce the major compounds of their glandular secretion de novo.

Not all phylogenetic relationships among the studied Pseudodineurini species could be resolved. However, it is obvious that *P. clematidisrectae* and *P. clematidis* are sister species, as are probably *P. parvula* and *P. enslini*. The interesting point is that in both species pairs, one species produces the 8-oxocitral isomers, the other does not. This implies that the production of 8-oxocitrals is not a synapomorphic trait for a certain subgroup of Pseudodineurini. We hypothesize that one or more gene(s) have been switched on/off several times during evolution. Citral might be converted by enzymatic reaction into 8-oxocitral, but it is more likely that geraniol or nerol is first enzymatically oxidized at the terminal position to the respective 8-hydroxygeraniol or 8-hydroxynerol, which is subsequently enzymatically oxidized to the dialdehyde. Since citral occurred in the secretion of all Pseudodineurini, obviously derived from geraniol by oxidation, the same precursor for 8-oxocitral is available in each species. Thus, the on/off switching that leads to the presence or absence of the 8-oxocitrals would require an additional enzyme, namely an oxidase that acts on geraniol, while the final oxidation might be performed by the same enzyme in the case of citral and 8-oxocitral. A potential subsequent step is the cyclization of 8-oxocitral into dolichodial. This is a major compound of the glandular secretion in several nematine sawflies (Boevé et al. 1984, 1992; Boevé and Heilporn 2009). Traces of iridoids such as dolichodial were detected in the glandular secretion of Pseudodineurini larvae, but it is not clear how they are formed. The pathway that involves two enzymatic steps from geraniol or nerol to the dialdehyde is described in leaf beetles, which use these dialdehydes as precursors of defensive iridoids (Veith et al. 1996). 8-Oxocitral is also the precursor of iridoids in plants.

What is the adaptive value of 8-oxocitral, and why does it occur in only some Pseudodineurini species? This monoterpene is inefficient as a defense against attacking small arthropods (Fig. 4), but it is an efficient fungus inhibitor as well as a fungicide (Table 2). Such antifungal activities would be relevant when the larva lives in a mine since feces accumulate therein (Fig. 1), offering a substrate for the proliferation of fungi. Additionally, when a full-grown larva crawls into the soil, it again may be subject to fungal infestation. Living in leaf mines and pupating in the soil are life history traits common to all Pseudodineurini larvae, and it does not explain why only some *Pseudodineura* species produce 8-oxocitral. There is, however, a parallel between an abiotic factor in the biotope where species live, as recorded in Altenhofer and Pschorn-Walcher (2006), and the presence vs. absence of 8-oxocitral. The humid and cold zones species *P. clematidis*, *P. enslini*, and *P. fuscula* produce 8-oxocitral, whereas the xerotherm species *P. clematidisrectae*, *P. heringi*, and *P. parvula* do not (P=0.05, Fisher exact probability test; N=6 species, not considering *P. mentiens* that showed variable chemical data, see below). We, thus, think that larvae crawling into a relatively warm and dry soil are less susceptible to fungal infestation, and that the production of 8-oxocitral may be less necessary. Indeed, entomopathogenic fungi are rarer in dry zones than in humid ones (Hall and Papierok 1982), and they require a high relative humidity, of at least 92–93%, for sporulation and for spore germination on insect adults and larvae (Deacon 1997). In this context, it is noteworthy that only the geographic distribution of *P. enslini*, *P. fuscula*, and *P. mentiens* extends into the humid cold zones of North Europe (Viramo 1969), and these species produce 8-oxocitral. Once the mining habit and the production of citral evolved in the ancestor of the Pseudodineurini, the production of 8-oxocitral may have been opted by those species living in relatively humid and cold zones.

We have no clear answer whether the adaptation to produce 8-oxocitral happened at a specific or subspecific level. Two samples of *P. fuscula* larvae were chemically

analyzed. The larvae came from two different populations, one collected on *R. bulbosus* and the other on *R. platanifolium*. 8-Oxocitral was detected in both samples. In contrast, larvae from one population of *P. mentiens* contained 8-oxocitral, whereas another sample did not. Interestingly, these latter larvae were collected from the xerotherm biotope (location "Langenlois"; Table 1). This suggests that, at least for *P. mentiens*, the production of 8-oxocitral is an adaptation at the population level. The intraspecific variation that we observed at the genetic level in several species should be studied more carefully to see whether it is linked to the (sub)-specific production of 8-oxocitral.

Our study began by asking why a larva that lives concealed and protected in a mine also would produce a defensive volatile secretion. By investigating the occurrence of citral in the larvae, the discrete occurrence of the biosynthetically related 8-oxocitral was incidentally detected. This stimulated us to reconstruct the phylogeny of the insect group and to compare the bioactivities of both compounds. It was surprising that 8-oxocitral was detected, twice, in only one of two closely related species. Further, the bioassays were at first restricted to ant repellence tests. We expected a higher activity with 8-oxocitral than citral since the former compound possesses two (reactive) aldehyde groups. When the bioassay results showed the opposite antifungal tests were performed due to the observation that feces accumulate in a leaf mine. These results led to the conclusion that abiotic factors play an important role in selecting for 8-oxocitral, since it is present in species that live in cold and humid areas. The efforts of a multidisciplinary team were necessary in order to integrate this accumulation of unpredictable results and to construct a more comprehensive picture of the evolution of a particular defense strategy in insects. Finally, M. Hering who devoted his life to leaf-mining insects mentions as an epigraph a sentence by J. Swammerdam that is strangely connected to our work: *Ich habe mir sagen lassen*, *in heißen Ländern fände man zwischen den Blättern daumenlange Würmer* (Hering 1935-1937), i.e., "I was informed that in warm countries one can find inch-long worms between the leaves."

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Fumigant and Contact Toxicities of Monoterpenes to *Sitophilus oryzae* (L.) and *Tribolium castaneum* (Herbst) and their Inhibitory Effects on Acetylcholinesterase Activity

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Abstract A comparative study was conducted to assess the contact and fumigant toxicities of eleven monoterpenes on two important stored products insects—, *Sitophilus oryzae*, the rice weevil, and *Tribolium castaneum*, the rust red flour beetle. The monoterpenes included: camphene, (+)-camphor, (−)-carvone, 1-8-cineole, cuminaldehyde, (L)-fenchone, geraniol, (−)-limonene, (−)-linalool, (−)-menthol, and myrcene. The inhibitory effect of these compounds on acetylcholinesterase (AChE) activity also was examined to explore their possible mode(s) of toxic action. Although most of the compounds were toxic to *S. oryzae* and *T. castaneum*, their toxicity varied with insect species and with the bioassay test. In contact toxicity assays, (−)-carvone, geraniol, and cuminaldehyde showed the highest toxicity against *S. oryzae* with LC_{50} values of 28.17, 28.76, and 42.08 μg/cm^2, respectively. (−)-Carvone (LC_{50}= 19.80 μg/cm^2) was the most effective compound against *T. castaneum*, followed by cuminaldehyde (LC_{50}= 32.59 μg/cm^2). In contrast, camphene, (+)-camphor, 1-8-cineole, and myrcene had weak activity against both insects (i.e., LC_{50} values above 500 μg/cm^2). In fumigant toxicity assays, 1-8-cineole was the most effective against *S. oryzae* and *T. castaneum* (LC_{50}=14.19 and 17.16 mg/l, respectively). Structure-toxicity investigations revealed that (−)-carvone—, a ketone—, had the highest contact toxicity against the both insects. 1-8-Cineole—, an ether—, was the most potent fumigant against both insects. In vitro inhibition studies of AChE from adults of *S. oryzae* showed that cuminaldehyde most effectively inhibited enzyme activity at the two tested concentrations (0.01 and 0.05 M) followed by 1-8-cineole, (−)-limonene, and (L)-fenchone. 1-8-Cineole was the most potent inhibitor of AChE activity from *T. castaneum* larvae followed by (−)-carvone and (−)-limonene. The results of the present study indicate that (−)-carvone, 1,8-cineole, cuminaldehyde, (L)-fenchone, and (−)-limonene could be effective biocontrol agents against *S. oryzae* and *T. castaneum*.

Keywords Monoterpenes · Contact toxicity · Fumigant toxicity · Acetylcholinesterase · *Sitophilus oryzae* · *Tribolium castaneum*

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Introduction

Monoterpenes are common essential oil constituents that provide the essence and the odor of a plant. Several hundred naturally occurring monoterpenes are known. They are biosynthesized from geranyl pyrophosphate, the ubiquitous acyclic C_{10} intermediate of the isoprenoid pathway (Windholz et al. 1983). Monoterpenes can be classified into two major groups: monoterpene hydrocarbons that include acyclic aliphatic, monocyclic aliphatic, and dicyclic aliphatic and oxygenated monoterpenes that include acyclic monoterpenoids, monocyclic monoterpenoids, and dicyclic

monoterpenoids. The latter group includes many alcohols, aldehydes, ketones, ethers, and acids (Templeton 1969).

Monoterpenes exert a wide spectrum of biological actions that are important in food chemistry, chemical ecology, and the pharmaceutical industry. Also, their insecticidal and herbicidal properties suggest their importance as potential pest control agents (Kohli et al. 1998; Isman 1999, 2000; Romagni et al. 2000). Monoterpenes possess acute contact and fumigant toxicity to insects (Shaaya et al. 1991; Grodnitzky and Coats 2002; Lee et al. 2004; Waliwitiya et al. 2005; Choi et al. 2006; Kordali et al. 2006; Samarasekera et al. 2008), repellent activity (Mason 1990; Watanabe et al. 1993), antifeedant activity (Hough-Goldstein 1990; Hummelbrunner and Isman 2001; Argandoña et al. 2002), as well as development and growth inhibitory activity (Gunderson et al. 1985; Karr and Coats 1992). These interesting biological activities are attributed to the physical–chemical properties of monoterpenes that include: (1) lipophilicity and low vapor pressure (resulting in their non-persistence in soil and limited leaching into ground water), (2) low mammalian toxicity, and (3) novel modes of action. As such, monoterpenes provide many prototypes for the synthesis of new pesticides (Dayan et al. 1999; Isman 2000).

Fumigant and contact toxicities of some monoterpenes on rice weevil adults, *Sitophilus oryzae* (L.) (Kim and Ahn 2001; Park et al. 2003) and the rust red flour beetle, *Tribolium castaneum* (Herbst) (Rice and Coats 1994; Prates et al 1998; Lee et al. 2001a, 2003, 2004) have been demonstrated. However, most of these studies were preliminary since the values of median lethal concentration (LC_{50}) for monoterpenes on both insects were not estimated. In addition, the inhibitory effect of monoterpenes on *S. oryzae* and *T. castaneum* acetylcholinesterase, a target enzyme of neurotoxic insecticides, were not reported.

Therefore, the aim of the present work was to study the comparative contact and fumingant toxicities of different classes of monoterpenes on *S. oryzae* and *T. castaneum*. The in vitro inhibitory effect of monoterpenes on acetylcholinesterase (AChE) activity was examined to explore the mode-of-action of these compounds and to elucidate structure–insecticidal activity relationships.

Materials and Methods

Chemicals The monoterpenes, camphene (95%), (+)-camphor (98%), (−)-carvone (98%), 1-8-cineole (99%), cuminaldehyde (98%), (L)-fenchone (98%), geraniol (98%), (−)-limonene (96%), (−)-linalool (95%), (−)-menthol (98%), and myrcene (90%) were purchased from Sigma–Aldrich Chemical Co., Steinheim, Germany. Chemical structures of test monoterpenes are shown in Fig. 1. Malathion (95%; American Cyanamid Co, USA) was used as a reference insecticide. Acetylthiocholine iodide (ATChI) and 5,5′-dithio-bis(2-nitrobenzoic) acid (DTNB) were purchased from Sigma–Aldrich Chemical Co., USA.

Test Insects All experiments were conducted in the laboratory with colonies of the rice weevil *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) and the rust red flour beetle *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). Colonies were maintained in our laboratory for over 10 years without exposure to insecticides. *S. oryzae* was reared on sterilized whole wheat. *T. castaneum* was reared on wheat flour mixed with yeast (10:1, *w*/*w*). Insect rearing and all experimental procedures were carried out at 26± 1°C, 65±5% RH, and a L/D regime of 12:12 h. Adult insects used in toxicity studies were 2-week post-emergence.

Contact Toxicity Assay The insecticidal activity of eleven monoterpenes against the adults of *S. oryzae* and *T. castaneum* was evaluated by direct contact application assay (Qi and Burkholder 1981; Broussalis et al. 1999). A series of concentrations of each was prepared in acetone. One milliliter of each concentration was applied on the bottom of a glass Petri dish (9 cm diam) to give a range of concentrations (0.01–500 μg/cm^2). Solvent was allowed to evaporate for 2 min prior to introduction of insects. Twenty adults of each insect were separately placed into Petri dishes. Control dishes were treated with acetone alone. All treatments were replicated four times. Malathion was used as a reference insecticide. Mortality percentages were recorded after 24 h of treatment, and LC_{50} values were calculated according to Finney (1971).

Fumigant Toxicity Assay The toxicity of monoterpene vapors was tested against *S. oryzae* and *T. castaneum* adults by using a modified fumigant toxicity assay as described by Huang et al. (2000). Glass jars (1 l) were used as fumigation chambers. Monoterepenes were applied to filter paper pieces (2×3 cm) attached to the undersurface of the screw caps of jars at: 1, 2.5, 5, 10, 15, 20, 30, 40, 60, 80, 90, and 100 mg. The inner side of the jar's neck was painted with Vaseline to prevent direct contact of insects with monoterpenes. Caps were screwed tightly onto the jars containing 20 insects of *S. oryzae* or *T. castaneum* in each one. Control insects were kept under the same conditions without monoterpenes. Four replicates of each treatment were set up. The number of dead insects was counted after 24 h of treatment, and the mortality percentages and LC_{50} values were calculated according to Finney (1971).

AChE Activity Assay S. oryzae adults and *T. castaneum* larvae (0.5 g) were separately homogenized in 5 ml of 0.1 M ice-cold phosphate buffer (pH 7.0) using a Teflon glass tissue

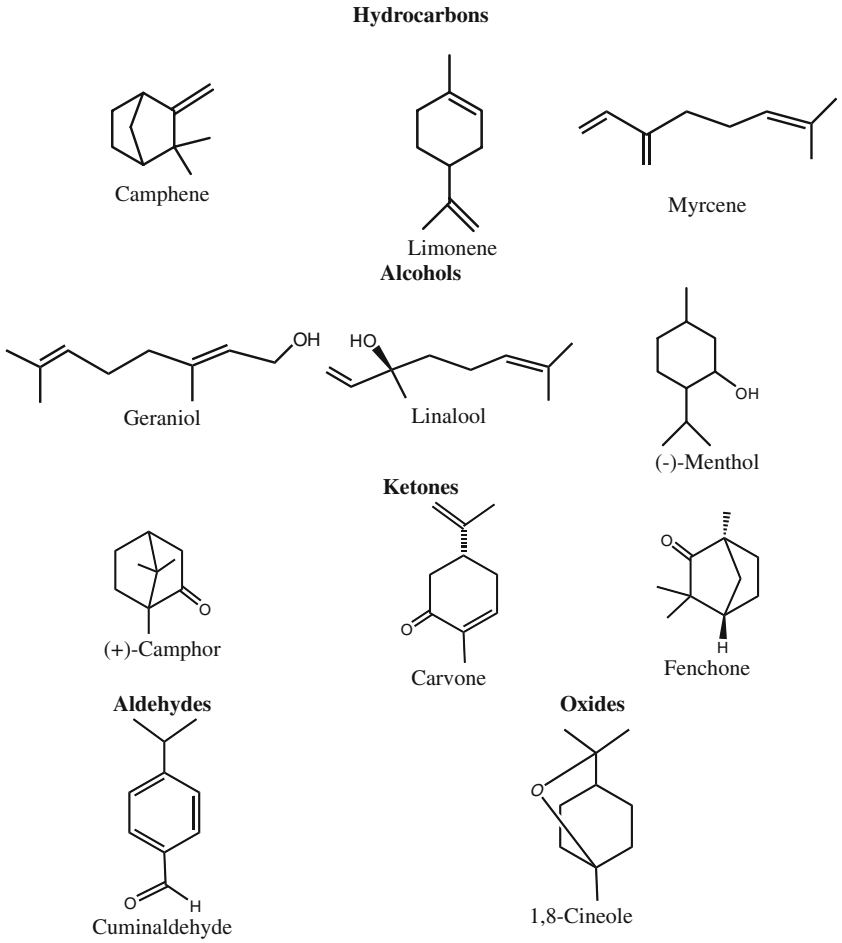

Fig. 1 The chemical structures of the monoterpenes tested

homogenizer. Homogenates were centrifuged (5,000 rpm for 20 min at 0ºC), and supernatants were used as the enzyme source for determination of AChE activity. Inhibition of AChE was determined by the colorimetric method of Ellman et al. (1961) using ATChI as substrate. Enzyme aliquots (50 µl) and DTNB (100 µl of 0.01 M) were added to 0.1 M phosphate buffer (pH 8.0; 2.8 ml). To this mixture, monoterpene test solutions (20 µl) prepared in dimethyl sulfoxide (DMSO) were added. Control treatments were prepared by the addition of DMSO (20 µl) in place of a monoterpene. Mixtures were incubated at 37ºC for 15 min. Reactions were started by adding ATChI (30 µl) followed by incubation at 37ºC for 10 min. Absorbance was measured at 412 nm using of Unico 1200-Spectrophotometer. Tested compounds were examined at two concentrations 0.01 and 0.05 M. Each test and control was corrected by blanks for nonenzymic hydrolysis. All the experiments were done in triplicate. Inhibition percentage of AChE activity was calculated as follows:

$$\text{AChE inhibition}(\%) = \frac{(OD_B - OD_T)}{OD_B} \times 100$$

where OD_B is the optical density of blank enzyme and OD_T is the optical density of treatment.

Statistical Analysis The concentration-mortality data were subjected to Probit analysis (Finney 1971) to obtain the LC_{50} values using the SPSS 12.0 software program (Statistical Package for Social Sciences, USA). The values of LC_{50} were considered to be significantly different, if 95% confidence limits did not overlap. The data of AChE inhibition was analyzed by one-way analysis of variance. Mean separations were performed by Student–Newman–Keuls (SNK) test and differences at $P<0.05$ were considered as significant.

Results and Discussion

Contact Toxicity The contact toxicity of 11 monoterpenes and a reference insecticide—malathion—was evaluated against *S. oryzae* and *T. castaneum* by using a residual film method. LC_{50} values, their 95% confidence limits expressed as microgram per square centimeter, and the slopes of toxicity regression lines are shown in Tables 1 and 2. (−)-Carvone and geraniol were the most effective among the monoterpenes tested against *S. oryzae* with LC_{50} values

of 28.17 and 28.76 μg/cm^2, respectively. Cuminaldehyde (an aldehyde monoterpene) and (−)-linalool (alcoholic monoterpene) also had relatively strong toxicities (LC_{50}= 42.08 and 66.74 μg/cm^2, respectively), while (−)-menthol (LC_{50}=221.68 μg/cm^2) and (L)-fenchone (LC_{50}= 291.80 μg/cm^2) elicited moderate toxic effects. Camphene and myrcene (hydrocarbon monoterpenes), (+)-camphor (a ketone monoterpene), and 1-8-cineole (an oxide monoterpene) had the lowest toxicity with LC_{50} values above 500 μg/cm^2. The alcoholic monoterpenes -, geraniol, (−)-linalool, and (−)-menthol—, possessed pronounced activity, whereas the hydrocarbon monoterpenes—, camphene, (−)-limonene, and myrcene—, were less active (Table 1).

Contact toxicity assays of monoterpenes on *T. castaneum* larvae shown in Table 2 revealed that (−)-carvone was the most effective compound, followed by cuminaldehyde and (−)-linalool, LC_{50} values of 19.80, 32.59, and 105.63 μg/cm^2, respectively. These results are similar to *S. oryzae* data (Table 1). (L)-Fenchone (a ketone monoterpene) and geraniol (alcoholic monoterpene) were of moderate toxicity against *T. castaneum* (LC_{50}=179.49 and 195.02 μg/cm^2, respectively). In contrast, camphene, (+)-camphor, 1-8-cineole, (−)-menthol, and myrcene had weak toxicity (i.e., LC_{50} values greater than 500 μg/cm^2).

Few studies have reported on monoterpene contact toxicities against *S. oryzae* and *T. castaneum*. For example, Park et al. (2003) found that treatment of *S. oryzae* adults with limonene and myrcene (260 μg/cm^2 after 2 days of contact) resulted in 36% and 10% mortality, respectively. This activity was comparable to the activity of limonene and myrcene reported here. The contact toxicities of fenchone to *S. oryzae* (Kim and Ahn 2001) and of 1-8-cineole and limonene to *T. castaneum* (Prates et al. 1998) have been reported. The activity found in this study for (−)-limonene, myrcene, and camphene on *T. castaneum* agrees with that reported by Garcìa et al. (2005), who stated that limonene had a moderate toxicity, while myrcene and camphene were inactive.

Fumigant Toxicity of Monoterpenes The toxicity of monoterpene vapors to *S. oryzae* and *T. castaneum* are summarized in Tables 3 and 4. Test compounds arranged by monoterpene class displayed varied toxicity against the two insects. 1-8-Cineole and (−)-carvone had the highest toxicity toward *S. oryzae* with LC_{50} values of 14.19 and 17.78 mg/l, respectively. (−)-Limonene, myrcene, and (L)-fenchone possessed lower fumigant activity (LC_{50}=26.92, 27.37, and 29.61 mg/l, respectively), while (−)-linalool, menthol, and cuminaldehyde had only moderate toxicity (LC_{50}=52.78, 61.75 and 71.40 mg/l, respectively). With LC_{50} values above 100 mg/l, camphene, (+)-camphor, and geraniol, were the least effective compounds.

Among monoterpenes tested against *T. castaneum,* 1-8-cineole had the lowest LC_{50} value of 17.16 mg/l (Table 4). The hydrocarbon monoterpenes myrcene and (−)-limonene had pronounced toxicity with LC_{50} values of 31.70 and 33.37 mg/l, respectively). Camphene, (+)-camphor, cuminaldehyde, geraniol, (−)-linalool, and (−)-menthol were

Table 1 Contact toxicity of monoterpenes against *Sitophilus oryzae* using residual film assay

Monoterpene class	Monoterpene	LC_{50}[a] (μg/cm^2)	95% Confidence limits (μg/cm^2)		Slope ± S.E.[b]	Intercept ± S.E.[c]	(χ^2)[d]
			Lower	Upper			
Hydrocarbons	Camphene	>500					
	(−)-Limonene	477.19	471.89	482.88	33.98±3.66	−91.03±9.80	1.12
	Myrcene	>500					
Alcohols	Geraniol	28.76	24.94	32.22	3.08±0.33	−4.50±0.53	1.27
	(−)-Linalool	66.74	57.08	76.55	8.44±0.73	−15.40±1.33	13.76
	(−)-Menthol	221.68	161.95	307.76	1.16±0.26	−2.72±0.61	0.26
Ketones	(+)-Camphor	>500					
	(−)-Carvone	28.17	26.75	29.70	8.46±0.82	−12.27±1.18	0.08
	(L)-Fenchone	291.80	235.53	335.67	9.24±0.89	−22.78±2.20	17.07
Aldehydes	Cuminaldehyde	42.08	40.09	44.04	6.88±0.67	−11.18±1.10	0.10
Oxides	1-8-Cineole	>500					
	Malathion	0.16	0.14	0.17	2.60±0.16	9.88±0.62	7.31

[a] The lethal concentration causing 50% mortality after 24 h

[b] Slope of the concentration-mortality regression line ± standard error.

[c] Intercept of the regression line ± standard error

[d] Chi square value

Table 2 Contact toxicity of monoterpenes against *Tribolium castaneum* using residual film assay

Monoterpene class	Compound	LC_{50}[a] (μg/cm^2)	95% Confidence limits (μg/cm^2)		Slope ± S.E.[b]	Intercept ± S.E.[c]	(χ^2)[d]
			Lower	Upper			
Hydrocarbons	Camphene	>500					
	(−)-Limonene	494.18	478.46	510.30	11.73±1.22	−31.60±3.31	0.40
	Myrcene	>500					
Alcohols	Geraniol	195.02	179.35	210.57	4.44±0.38	−10.17±0.88	1.34
	(−)-Linalool	105.63	97.21	121.55	4.72±0.59	−9.56±1.21	1.46
	(−)-Menthol	>500					
Ketones	(+)-Camphor	>500					
	(−)-Carvone	19.80	11.63	35.97	2.77±0.24	−3.59±0.31	5.79
	(L)-Fenchone	179.49	153.39	205.30	2.73±0.31	−6.15±0.73	0.75
Aldehydes	Cuminaldehyde	32.59	23.39	40.62	5.30±0.61	−8.02±0.94	4.25
Oxides	1-8-Cineole	>500					
	Malathion	3.88	2.61	5.50	2.34±0.18	5.65±0.44	17.87

[a] The lethal concentration causing 50% mortality after 24 h

[b] Slope of the concentration-mortality regression line ± standard error

[c] Intercept of the regression line ± standard error

[d] Chi square value

significantly less toxic with LC_{50} values greater than 100 mg/l.

The fumigant toxicity of some of the test monoterpenes (i.e., camphor, 1-8-cineole, geraniol, limonene, and linalool) have been examined previously against *S. oryzae* (Lee et al. 2001a, 2003). The toxicity of these compounds was in general agreement with the data presented in this report. However, 1-8-cineole, limonene, and fenchone also possessed fumigant toxicity against *T. castaneum* (Prates et al. 1998; Lee et al. 2003, 2004).

Toxicity of the test monoterpenes varied with insect species and bioassay method. For example, in contact assays, geraniol, (−)-linalool, and (−)-menthol were more toxic to *S. oryzae,* whereas (−)-carvone, cuminaldehyde,

Table 3 Fumigant toxicity of monoterpenes against the adults of *Sitophilus oryzae*

Monoterpene class	Monoterpene	LC_{50}[a] (mg/l)	95% Confidence limits (mg/l)		Slope ± S.E.[b]	Intercept ± S.E.[c]	(χ^2)[d]
			Lower	Upper			
Hydrocarbons	Camphene	>100					
	(−)-Limonene	26.92	20.87	30.37	4.36±0.91	−6.23±1.44	1.47
	Myrcene	27.37	2.39	40.60	4.96±0.48	−7.14±0.73	9.82
Alcohols	Geraniol	>100					
	(−)-Linalool	52.78	42.18	69.98	1.35±0.19	−2.32±0.30	3.09
	(−)-Menthol	61.75	52.46	73.56	2.38±0.29	−4.25±0.49	0.15
Ketones	(+)-Camphor	>100					
	(−)-Carvone	17.78	8.12	35.11	3.47±0.25	−4.32±0.32	31.60
	(L)-Fenchone	29.61	19.72	38.14	6.20±0.54	−9.13±0.81	7.10
Aldehydes	Cuminaldehyde	71.40	66.99	75.92	5.90±0.68	−10.93±1.26	1.74
Oxides	1-8-Cineole	14.19	12.88	15.51	4.71±0.48	−5.42±0.58	0.10

[a] The lethal concentration causing 50% mortality after 24 h

[b] Slope of the concentration-mortality regression line ± standard error

[c] Intercept of the regression line ± standard error

[d] Chi square value

Table 4 Fumigant toxicity of monoterpenes against the adults of *Tribolium castaneum*

Monoterpene class	Compound	LC_{50}[a] (mg/l)	95% Confidence limits (mg/l)		Slope ± S.E.[b]	Intercept ± S.E.[c]	(χ^2)[d]
			Lower	Upper			
Hydrocarbons	Camphene	>100					
	(−)-Limonene	33.37	31.85	34.90	7.76±0.64	−11.82±0.98	3.44
	Myrcene	31.70	30.18	33.37	7.86±0.81	−11.79±1.21	0.44
Alcohols	Geraniol	>100					
	(−)-Linalool	>100					
	(−)-Menthol	>100					
Ketones	(+)-Camphor	>100					
	(−)-Carvone	75.22	67.42	85.84	2.86±0.35	−5.37±0.63	2.63
	(L)-Fenchone	43.35	41.59	45.39	9.05±1.02	−14.81±1.66	0.42
Aldehydes	Cuminaldehyde	>100					
Oxides	1-8-Cineole	17.16	12.12	20.24	3.23±0.67	−3.98±0.96	1.05

[a] The lethal concentration causing 50% mortality after 24 h

[b] Slope of the concentration-mortality regression line ± standard error

[c] Intercept of the regression line ± standard error

[d] Chi square value

and (L)-fenchone were more effective on *T. castaneum*. Camphene, camphor, 1-8-cineole, (−)-limonene, and myrcene had approximately the same toxicity against both insects. Most test compounds in fumigant assays were more effective toward *S. oryzae* than *T. castaneum,* except for camphene, camphor, and geraniol that had weak activity toward both insects. Differential toxicities to *S. oryzae* and *T. castaneum* by monoterpenes have been noted previously (Lee et al. 2003, 2004).

Assay method impacted the toxicity of monoterpenes. For example, 1-8-cineole and myrcene had pronounced fumigants toxicity toward *S. oryzae* but were weak contact toxicants. In contrast, geraniol was an effective contact toxicant against *S. oryzae*, but was a weak fumigant. With *T. castaneum*, 1-8-cineole and myrcene were among the most toxic compounds in fumigant assays, but the two compounds were only weakly toxic in contact assays. Cuminaldehyde, geraniol, and (−)-linalool had strong contact toxicity but weak fumigant activity, while (−)-limonene showed the reverse. It is clear that assay method impacts the toxicity and efficacy of the test monoterpenes. Similar conclusions have been reported regarding the insecticidal activity of monoterpenes against stored product insects (Prates et al. 1998; Park et al. 2003).

The contact and fumigant toxicities of various monoterpenes and how activity varies with structure was investigated. (−)-Carvone, a ketone, showed the highest contact toxicity, while an aldehyde (cuminaldehyde) and an alcohol ((−)-linalool) were more toxic than other oxygenated and non-oxygenated monoterpenes. In contrast, 1-8-

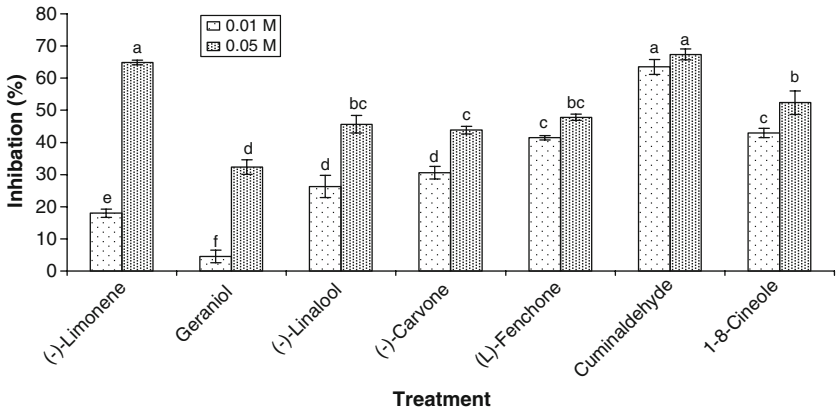

Fig. 2 In vitro inhibition of *Sitophilus oryzae* adult acetylcholinesterase activity by monoterpenes. Data are means ± SE of three replicates. *Different letters* are significantly different at 0.05 level

cineole, an ether-containing monoterpene, was the most potent fumigant. This finding agrees with Lee et al. (2003) who stated that 1-8-cineole was more toxic than some ketone compounds. Two hydrocarbons—, (−)-limonene (a monocyclic monoterpene) and myrcene (an acyclic monoterpene) were among the most active fumigant toxicants. (−)-Carvone and (L)-fenchone (both ketones) were more effective than either of the alcohols, i.e., (−)-linalool and geraniol. Similarly, Rice and Coats (1994) found that some ketones were more effective fumigants than alcohols. These observations raise the possibility that the presence of a carbonyl group augments toxicity.

Inhibitory Effect of Monoterpenes on AChE Activity The in vitro inhibitory effect of monoterpenes on AChE from adults of *S. oryzae* and larvae of *T. castaneum* was examined at 0.01 and 0.05 M (Figs. 2 and 3). Cuminaldehyde was the most effective inhibitor of *S. oryzae* AChE activity (63.50% and 67.35% at 0.01 and 0.05 M, respectively), followed by 1-8-cineole (42.96% and 52.37% at 0.01 and 0.05 M, respectively), and (−)-limonene (64.86% at 0.05 M). In contrast, geraniol was the least effective inhibitor of *S. oryzae* AChE at the two concentrations (4.51% and 32.33% at 0.01 and 0.05 M, respectively).

The inhibitory effects of monoterpenes on AChE derived from *T. castaneum* larvae indicated that a 1-8-cineole had the greatest inhibitory activity and there was no significant difference between the two concentrations (64.90% and 70.53% at 0.01 and 0.05 M, respectively). This was followed by (−)-carvone (56.79% and 70.20% inhibition at 0.01 and 0.05 M, respectively) and (−)-limonene (42.63% and 51.66% at 0.01 and 0.05 M, respectively).

Our results agree with the reports of others regarding the inhibitory activity of monoterpenes on AChE activity. Of four monomterpenes (1-8-cineole, (−)-carvone, (−)-limonene, or (−)-linalool, 1,8-cineole was the most inhibitory toward electric eel AChE activity (Picollo et al. 2008). In addition, monoterpenes have been shown to be effective inhibitors of AChE's from eel, housefly, and bovine erythrocyte (Gracza 1985; Grundy and Still 1985; Ryan and Byrne 1988; Miyazawa et al. 1997).

Comparing monoterpene toxicity against *S. oryzae* with the inhibitory effects on AChE activity from the same insect show that some of the monoterpenes possess strong insecticidal activity and potent AChE inhibitory activity—, e.g., cuminaldehyde, 1-8-cineole, (−)-limonene, and (L)-fenchone. However, other compounds (e.g., (−)-carvone and geraniol) have strong insecticidal activity but are weak inhibitors of AChE. Similarly, Lee et al. (2001b) did not find a direct correlation between insect toxicity and AChE inhibition by menthone or β-pinene. These findings suggest that AChE may be a target for monterpenes but does not rule out that there may be other targets.

The development of natural insecticides would help to decrease the negative impact of synthetic insecticides, such as residues, resistance, and environmental pollution. In this respect, natural insecticides may be effective, selective, biodegradable, and less harmful to the environment. In the present study, monoterpenes such as (−)-carvone, 1,8-cineole, cuminaldehyde, (L)-fenchone, and (−)-limonene showed strong contact and/or fumigant toxicities against *S. oryzae* and *T. castaneum*. Based on these findings, these or other monoterpenes may serve as viable alternatives to synthetic insecticides.

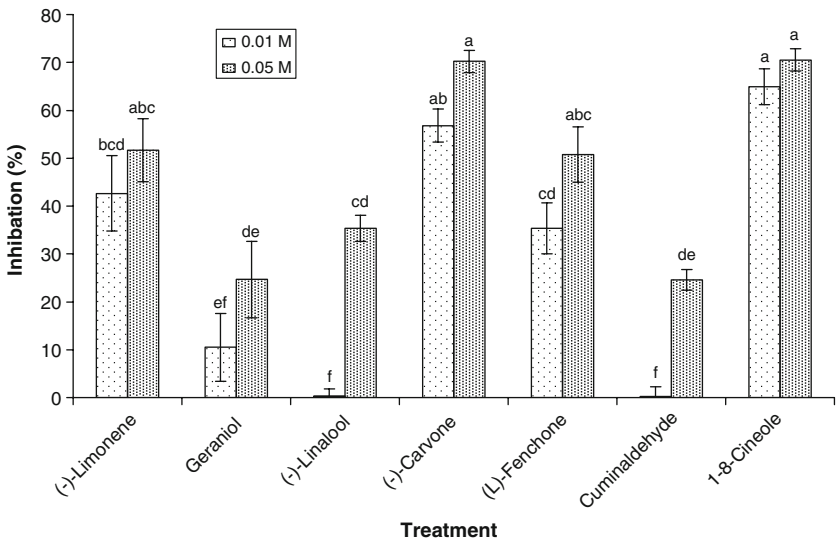

Fig. 3 In vitro inhibition of *Tribolium castaneum* larvae acetylcholinesterase by monoterpenes. Data are means ± SE of three replicates. *Different letters* are significantly different at 0.05 level

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Enhanced Toxicity and Induction of Cytochrome P450s Suggest a Cost of "Eavesdropping" in a Multitrophic Interaction

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Abstract The inducibility of cytochrome P450 monooxygenases (P450s) and other xenobiotic-metabolizing enzymes is thought to reflect material and energy costs of biosynthesis. Efforts to detect such costs of detoxification enzyme induction, however, have had mixed success. Although they are rarely considered, ecological costs of induction may be a more significant evolutionary constraint on herbivores than material and energy costs. Because some P450-mediated metabolic transformations are bioactivation reactions that increase, rather than reduce, toxicity, maintaining high levels of P450 activity places an organism at risk of greater mortality in the presence of compounds that are bioactivated. We show that P450 inducibility in the generalist moth *Helicoverpa zea* in response to plant signaling substances, an adaptive response in a ditrophic interaction between herbivore and plant, becomes detrimental in the presence of a third trophic association with a plant pathogen that produces aflatoxin, a toxin that can be bioactivated by P450s. Consumption of plant signaling molecules, such as methyl jasmonate (MeJA) and salicylic acid (SA) enhanced the toxicity of aflatoxin B1 (AFB1) to *H. zea* that resulted in substantially more damage to larval growth and development. Among the P450 transcripts already cloned from this organism, two in the CYP6B and CYP321A subfamilies are shown to be induced in response to MeJA and SA, suggesting that they may mediate some of the observed bioactivations.

Keywords Aflatoxin B1 · Bioactivation · Cytochrome P450 monooxygenase · Detoxification induction · Ecological cost · *Helicoverpa zea* · Multitrophic interaction · Plant signaling molecule · RT-PCR · Southern analysis

Introduction

As primary producers in most terrestrial trophic webs, plants are at risk of attack by a broad spectrum of consumers, including both pathogens and herbivores, and their principal defense is the production of toxic compounds. Different suites of defense genes are activated in response to potential consumers via several interacting signaling pathways in plants. In a wide variety of species, mechanical damage, including that inflicted by chewing herbivores, activates many genes that are responsive to jasmonic acid (JA) and related octadecanoids, including methyl jasmonate (MeJA) and 12 oxophytodienoic acid (OPDA) (Kessler and Baldwin 2002; Moran and Thompson 2001; Reymond et al. 2000, 2004). Feeding damage, along with insect-specific elicitors such as volicitin, a fatty acid conjugate, induces the synthesis of octadecanoid signals, which in turn can trigger production of toxins and antinutritive substances that deter further herbivory (Pechan et al. 2000; Lou and Baldwin 2004; Mewis et al. 2005). Similarly, infection by pathogens in many plant species leads to induction of salicylic acid, which in turn induces broad-spectrum protection against subsequent pathogen

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infection through a variety of mechanisms (Yang et al. 1997; De Vos et al. 2005). Although mechanical damage does not lead to increased production of salicylic acid (SA) (DeMoraes et al. 2004), insect herbivory is in some cases associated with elevated SA production (Bi et al. 1997), and, because salicylate can inhibit JA production (Harms et al. 1998), cross-talk and interactions among these signaling pathways collectively determine patterns of plant chemical defense responses to herbivory.

Helicoverpa zea (Boddie) (Lepidoptera: Noctuidae) is a highly polyphagous caterpillar that utilizes over 100 plant species as hosts (Wiseman 1999). In this species, cytochrome P450s (P450s) are responsible for metabolizing a broad range of the allelochemicals encountered in the diet. Heterologous expression of individual P450s demonstrated that at least two of the P450s from this species, CYP6B8 and CYP321A1, are broadly substrate-specific (Li et al. 2004; Sasabe et al. 2004). That CYP6B8 transcripts can be induced by ingestion of JA suggests that *H. zea* can "eavesdrop" on the signaling systems of plants by responding to these signals via upregulation of particular sets of P450s prior to the biosynthesis of defense chemicals in plants (Li et al. 2002b).

Infestation by *H. zea* predisposes some plants to infection by fungi, particularly the *Aspergillus* ear molds that produce aflatoxins (Lillehoj 1992; Dowd 1998), dihydrofurocyclopentenones and dihydrofurolactones that form irreversible adducts to nucleic acids with concomitant inhibition of DNA replication and DNA-dependent transcription (Murray et al. 1982; Iyer et al. 1994). In vertebrates, some P450s catalyze the epoxidation of the terminal furan ring of aflatoxin B1 (AFB1) to yield AFB1-8,9-epoxide (AFBO), a highly genotoxic metabolite (Murray et al. 1982). In the Hikone-R strain of the fruit fly, *Drosophila melanogaster*, bioactivation of AFB1 is catalyzed by CYP6A2 (Saner et al. 1996). In contrast, however, the caterpillar *Amyelois transitella* (navel orangeworm), which frequents fungus-contaminated fruit tissue, produces aflatoxicol (AFL) as the major aflatoxin metabolite and does not produce AFBO as a metabolite at all, thus leading Lee and Campbell (2000) to suggest that the absence of this particular biotransformation is indicative of coevolution between kernel-feeding insects and toxin-producing fungi.

Helicoverpa zea larvae do not regularly encounter aflatoxins across their many hosts, and they are highly sensitive to low concentrations of these mycotoxins in their diet (Dowd 1988; Zeng et al. 2006), which suggests that P450-mediated metabolism of aflatoxins may result in bioactivation rather than detoxification in this species. Because "eavesdropping" on plant signaling systems results in increased transcription of particular sets of P450s in *H. zea* (Li et al. 2002b), we examined the transcript profiles for several P450s previously cloned in this species (Li et al. 2002a, b; Sasabe et al. 2004) in response to signaling substances and aflatoxin B1. We show here that P450 inducibility in *H. zea* in response to plant signaling substances appears to be detrimental, rather than beneficial, in the presence of a mycotoxin, which is produced as a consequence of an additional trophic association between a plant and its phytopathogen.

Methods and Materials

Chemicals Aflatoxin B1 (AFB1), methyl jasmonate (MeJA), and salicylic acid (SA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). RQ1 DNase, RNasin, and AMV reverse transcriptase were from Promega (Madison, WI, USA), Taq polymerase and deoxynucleotide triphosphates (dNTPs) were from Invitrogen (Carlsbad, CA, USA), and the TRIzol reagent was from Gibco-BRL (Cleveland, OH, USA).

Insects A laboratory colony of *H. zea*, initiated from caterpillars collected in Champaign County, IL in sweet corn, was used for all studies. *H. zea* larvae were reared individually on a semi-synthetic control diet containing wheat germ (Waldbauer et al. 1984), and maintained in an insectary kept at 23–26°C with a photoperiod of 16:8 h | L:D. Caterpillars were collected within 12 h of new molting to ensure that they were homogeneous in their developmental age.

Bioassays Stock solutions of AFB1 were prepared at a concentration of 1 µg/µl in DMSO, SA was prepared at concentrations of 1 mg/µl and 12 µg/µl, and MeJA was prepared at concentrations of 100 µg/µl and 2.9 µg/µl, all in DMSO; these solutions were included in diets with final concentrations of DMSO not exceeding 0.2%. Newly molted fourth and fifth instars were assayed with 20 larvae per treatment, with two sets of experiments performed for each developmental stage. In the first set of SA experiments, larave were placed on diets containing either 0.2% DMSO (control), SA (1 mg/g, High SA [H-SA]; or 12 µg/g, Low SA [L-SA]), 1 µg/g AFB1 or [1 µg/g AFB1 + H-SA or L-SA]. In the second set of MeJA experiments, larvae were provided with diets containing either 0.2% DMSO (control), MeJA (100 µg/g, High MeJA [H-MeJA]; or 2.9 µg/g, Low MeJA [L-MeJA]), 1 µg/g AFB1 or [1 µg/g AFB1 + H-MeJA or L-MeJA]. Larvae exposed to the four different diets in each set of experiments were individually reared in 30-ml plastic cups in an insectary at 23–26°C with a photoperiod of 16:8 h | L:D. For fourth instars, mortality was recorded after 8 d. For fifth instars, weight gain was

measured after 3 d of treatments, and pupation was recorded every day. For individuals surviving through pupation, pupal weights were measured within 24 h of pupation. Each set of bioassays was independently replicated three times.

Statistical Analyses All data were evaluated by two-way factorial analysis of variance (ANOVA) with treatment differences among means tested at $P = 0.05$ by Tukey *post-hoc* test. Data for mortality are the means from three replicates with 20 larvae/treatment after 8 d of treatments.

Transcript Analysis Fifth instar *H. zea* larvae molting within a period of 12 h and within a weight range of about 170–270 mg were grouped randomly. Twenty larvae in each treatment group were switched to diets containing the plant signaling chemicals at the following concentrations: MeJA at 2.9 μg/g diet and 100 μg/g diet, and SA at 12 μg/g diet and 1 mg/g diet. The control was set with caterpillars feeding on a diet containing 0.2% DMSO because this solvent was used for each of the signaling chemicals added to diets. Forty-eight hours after feeding, larval midguts were excised and cleaned in 0.1 M sodium phosphate buffer (pH 7.8). Total RNA was extracted from each group of twenty pooled larvae by using TRIzol reagent treated with 5 U RQ1 DNase for 30 min at 37°C, cleaned up with a Qiagen RNeasy kit (Valencia, CA, USA), quantified by A_{260}, and stored at −80°C.

RT-PCR Southern Analysis RT-PCR Southern analyses were used to measure semi-quantitatively the induction of the *CYP6B8* and *CYP321A1* transcripts. To keep RT-PCR signals within their linear ranges, amplification conditions for P450 and actin (control) transcripts were checked by varying PCR cycle numbers and RNA amounts to achieve conditions not limited by any of the components within these reactions. The final 50 μl RT-PCR reaction mixtures contained 100 ng total RNA, 0.5 μl RNasin (40 U/μl), 0.5 μl AMV reverse transcriptase (10 U/μl), 0.25 μl Taq polymerase (5 U/μl), 25 μl 2x RT buffer containing dNTPs and 1 μl (10 μM) of each forward and reverse primer. The gene-specific forward/reverse primer sets used in these reactions were: 5′-TCTTGTGGACAGCATTATTAGC-3′ and 5′-CAAGTCCGAATGGTAAGTACGC-3′ for *CYP6B8*, 5′-TAGTGTGGAGGGTGACCAACTG-3′ and 5′-CGGACAACAAGCCAGTCGTAGGC-3′ for *CYP321A1*, and 5′-TGGGAYGAYATGGAGAAGATCTGG-3′ and 5′-TAGATGGGBACBGTGTGBGAGACA-3′ (with Y (C/T) and B (C/G/T)) for actin. The RT-PCR reactions were initiated with a reverse transcription step at 42°C for 50 min, followed by a denaturation step at 94°C for 5 min, 18 cycles of PCR amplification (94°C for 1 min, 60°C for 1 min, 72°C for 2 min) and a final extension step at 72°C for 5 min. Control reactions conducted with cloned CYP6B8 and CYP321A1 templates indicated that, under these conditions, these primer sets were completely gene-specific. For their final quantification, 10 μl of concentrated (6x) 40% sucrose loading dye were added to each reaction, and 20 μl of each PCR product mixture were run on a 1.0% agarose gel containing 1x TBE buffer. After gel-blotting, the Hybond-N membranes (Amersham-Pharmacia Biosciences, Uppsala, Sweden) were pre-hybridized and then hybridized with ^{32}P-labeled gene-specific probes at 42°C overnight in hybridization buffer containing 5x SSC, 5x Denhardt's solution, 50% formamide, 50 mM sodium phosphate (pH 7.0), and 0.5% SDS. Membranes were washed twice at low stringency in buffer containing 2x SSC, 0.1% SDS at 42°C each for 15 min and once at high stringency in buffer containing 0.2x SSC, 0.1% SDS at 62°C for 15 min. Finally, the membranes were autoradiographed and quantified by phosphoimager analysis by using a Typhoon 8600 variable model imager (Amersham-Pharmacia Biotech).

Results and Discussion

To determine the effects of encountering aflatoxin in the presence of plant signaling substances, two sets of experi-

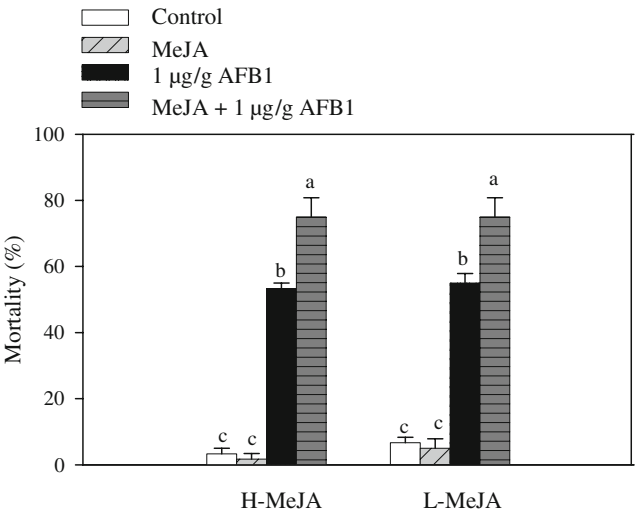

Fig. 1 Effects of methyl jasmonate (MeJA) on mortality of fourth instar *Helicoverpa zea* exposed to 1 μg/g aflatoxin B1 (AFB1) after 8 d. Caterpillars were reared on the diets containing either 0.2% DMSO (control), MeJA (100 μg/g, H-MeJA; or 2.9 μg/g, L-MeJA), 1 μg/g AFB1, or [1 μg/g AFB1 + H-MeJA or L-MeJA]. Values are means + standard error from three replicates with 20 caterpillars/treatment. Significant differences ($P < 0.05$ using Tukey *post-hoc* test) among treatments in a group are indicated by different letters above bars. Two-way ANOVA showed that effects of H-MeJA ($F = 9.6$, $P = 0.015$), AFB1 ($F = 365.1$, $P < 0.001$), and the H-MeJA×AFB1 ($F = 13.1$, $P = 0.007$) interaction were significant, and effects of L-MeJA ($F = 6.368$, $P = 0.036$), AFB1 ($F = 265.3$, $P < 0.001$), and the L-MeJA×AFB1 ($F = 8.895$, $P = 0.017$) interaction were significant

ments were performed on both fourth and fifth instars. In one set, larave were fed diets containing AFB1 in the presence and absence of the signaling substance MeJA; in the second set, larvae received artificial diets containing AFB1 in the presence and absence of the signaling substance SA. The signaling substances were incorporated into diets at low concentrations (MeJA at 2.9 µg/g and SA at 12 µg/g) approximating those produced by *H. zea* host plants as a consequence of herbivory or fungal infection (Seskar et al. 1998; Royo et al. 1999). High concentrations of the signaling compounds were selected to maximize the likelihood of detecting an upper limit on the response.

Although neither signaling substance alone in the diet had any detectable effect on fourth instar larvae for the duration of the bioassay, aflatoxin at a concentration of 1 µg/g affected mortality ranging from 35 to 55%. Both signaling substances further increased this toxicity. Inclusion of 100 µg/g MeJA (H-MeJA) and 2.9 µg/g MeJA (L-MeJA) in the diet increased mortality by 21.7% ($P = 0.006$) and 20%, respectively, compared with larvae exposed only to 1 µg/g AFB1 (Fig. 1). Exposure of fourth instars to diets containing 1 mg/g SA + 1 µg/g AFB1 and 12 µg/g SA + 1 µg/g AFB1 resulted in increases in mortality by 23.3% ($P = 0.021$) and 16.7% ($P = 0.141$) , respectively, compared with larvae exposed only to 1 µg/g AFB1 (Fig. 2).

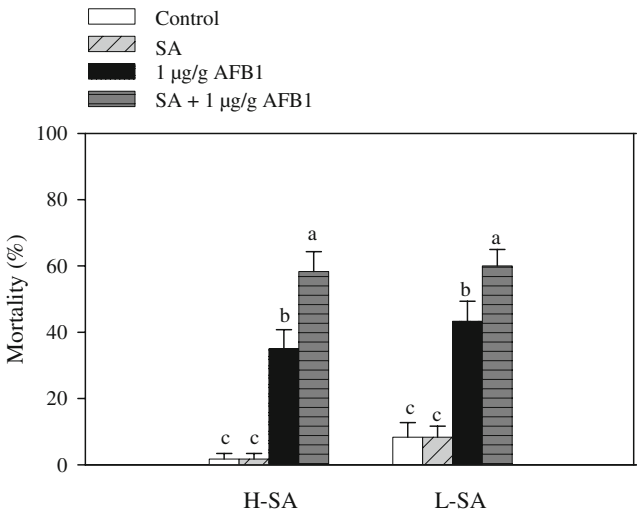

Fig. 2 Effects of salicylic acid (SA) on mortality of fourth instar *Helicoverpa zea* exposed to 1 µg/g aflatoxin B1 (AFB1) after 8 d. Caterpillars were reared on the diets containing either 0.2% DMSO (control), SA (1 mg/g, H-SA; or 12 µg/g, L-SA), 1 µg/g AFB1, or [1 µg/g AFB1 + H-SA or L-SA]. Values are means + standard error from three replicates with 20 caterpillars/ treatment. Significant differences ($P < 0.05$ using Tukey *post-hoc* test) among treatments in a group are indicated by different letters above bars. Two-way ANOVA showed that effects of H-SA ($F = 7.295$, $P = 0.027$), AFB1 ($F = 108$, $P < 0.001$), and the H-SA×AFB1 interaction ($F = 7.259$, $P = 0.027$) were significant, effects of L-SA ($F = 3.030$, $P = 0.119$) and the L-SA×AFB1 interaction ($F = 3.030$, $P = 0.120$) were not significant, the effect of AFB1 ($F = 81.939$, $P < 0.001$) was significant

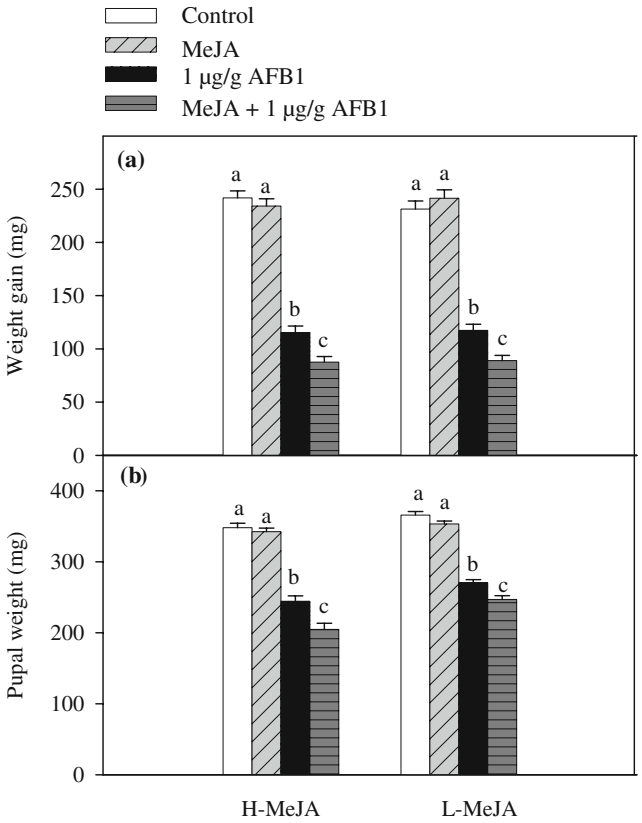

Fig. 3 Effects of methyl jasmonate (MeJA) on (a) weight gain after 3 d and (b) weight within 24 h of pupation for surviving larvae of fifth instar *Helicoverpa zea* exposed to 1 µg/g aflatoxin B1 (AFB1). Larvae were reared on the diets containing either 0.2% DMSO (control), MeJA (100 µg/g, H-MeJA; or 2.9 µg/g, L-MeJA), 1 µg/g AFB1, or [1 µg/g AFB1 + H-MeJA or L-MeJA]. Values for weight gain and pupal weight are means + standard error from three replicates with 20 larvae/ treatment. Significant differences ($P < 0.05$ using Tukey *post-hoc* test) among treatments in a group are indicated by different letters above the bars

An identical pattern was observed in bioassays with fifth instars. Fifth instar larave experienced declines in weight gain and pupal weight of 24.1% ($P = 0.011$) and 16.3% ($P < 0.001$), respectively, in the combined treatment of 100 µg/g MeJA + 1 µg/g AFB1 compared to treatment with 1 µg/g AFB1 alone (Fig. 3a,b). Inclusion of 2.9 µg/g MeJA in the AFB1 containing diet led to significant reduction in weight gain (24.3%, $P = 0.0179$) and pupal weight (8.8%, $P = 0.0016$) compared with larvae exposed only to 1 µg/g AFB1 (Fig. 3a, b). Exposure of fifth instar *H. zea* to diets containing 1 mg/g SA + 1 µg/g AFB1 resulted in a significant reduction in weight gain (36.1%, $P < 0.001$) and pupal weight (13.9%, $P < 0.001$) relative to larvae exposed only to 1 µg/g AFB1 (Fig. 4a, b). Similarly, exposure of fifth instars to diets containing 12 µg/g SA + 1 µg/g AFB1 resulted in significant reduction in weight gain (31.9%, $P < 0.001$) and pupal weight (13.7%, $P < 0.001$) compared with larvae exposed only to 1 µg/g AFB1 (Fig. 4a, b).

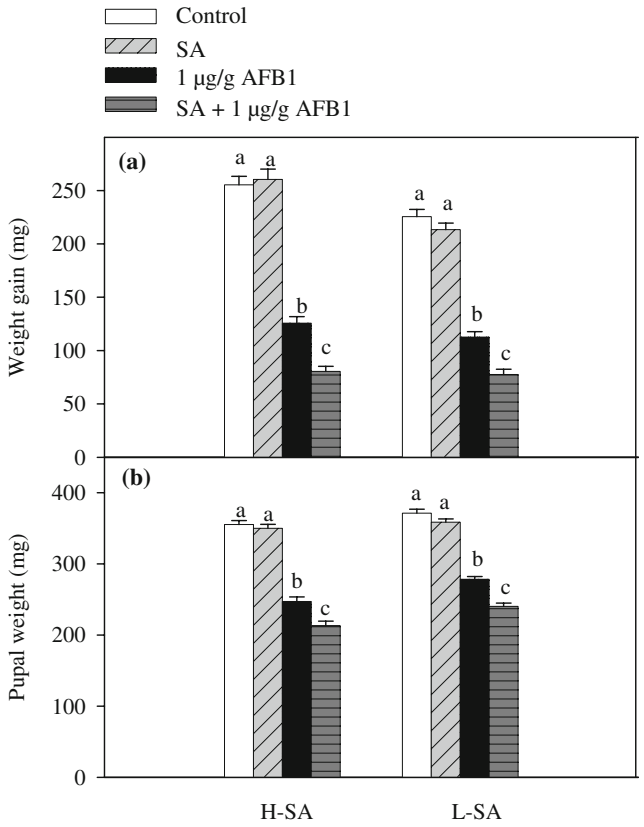

Fig. 4 Effects of salicylic acid (SA) on (a) weight gain after 3 d and (b) weight within 24 h of pupation for surviving larvae of fifth instar *Helicoverpa zea* exposed to 1 µg/g aflatoxin B1 (AFB1). Larvae were reared on the diets containing either 0.2% DMSO (control), SA (1 mg/g, H-SA; or 12 µg/g, L-SA), 1 µg/g AFB1, or [1 µg/g AFB1 + H-SA or L-SA]. Values for weight gain and pupal weight are means + standard error from three replicates with 20 larvae/treatment. Significant differences ($P < 0.05$ using Tukey *post-hoc* test) among treatments in a group are indicated by different letters above the bars

To determine the contributions of P450 upregulation to the increased toxicity of aflatoxin in the presence of signaling substances, transcript profiles for two P450s previously cloned in this species (Li et al. 2002a, b; Sasabe et al. 2004) were defined. RT-PCR analyses (Fig. 5) demonstrate that the *CYP6B8* and *CYP321A1* transcripts are induced to some extent in the presence of these plant signaling substances. Methyl jasmonate and salicylate induce *CYP6B8* and *CYP321A1* transcripts at least 2.1-fold. As mentioned, CYP6B8 and CYP321A1 are broadly substrate-specific enzymes (Li et al. 2004; Sasabe et al. 2004).

Bioactivation of aflatoxins by P450s has been demonstrated as a necessary step in bringing about most of their toxic effects in humans and other animals (Eaton et al. 1994). To date, no epoxide metabolite of aflatoxin has been identified in *H. zea*, although CYP321A1 expressed heterologously converts aflatoxin to aflatoxin P1, its O-demethylated product (Niu et al. 2008). The involvement of P450s in the bioactivation of aflatoxin in *H. zea* is supported by the significant decrease in the toxicity of AFB1 when the P450 inhibitor piperonyl butoxide (PBO) is included in the assay (Zeng et al. 2006), a result that is consistent with bioactivation of aflatoxin to more toxic derivatives by one or more P450s expressed in fourth and fifth instars. Molecular modeling data predict that aflatoxin can fit in the catalytic sites of CYP6B8 (Niu et al. 2008), which may be responsible for the bioactivation of aflatoxin in response to MeJA and SA.

Although evidence in support of a metabolic cost of inducing detoxification enzymes has been inconsistent (Neal 1987; Appel and Martin 1992; Cresswell et al. 1992; Kelley et al. 2002; Agrawal et al. 2002), this study suggests that "eavesdropping," or up-regulation of insect detoxification enzymes prior to plant biosynthesis of defense compounds, can exact a cost in situations where the defense compounds are bioactivated and not detoxified by P450s. The number of plant allelochemicals known to be bioactivated by P450-mediated reactions is limited to several classes that have relatively restricted distributions among plant families. Pyrrolizidine alkaloids, for example, which are bioactivated by P450s (Brattsten 1979), are effectively restricted to the Asteraceae, Fabaceae, and Boraginaceae (Seigler 1998; Stegelmeier et al. 1999). Thus, P450s of herbivorous insects up-regulated by plant signaling substances are likely to catalyze metabolic reactions

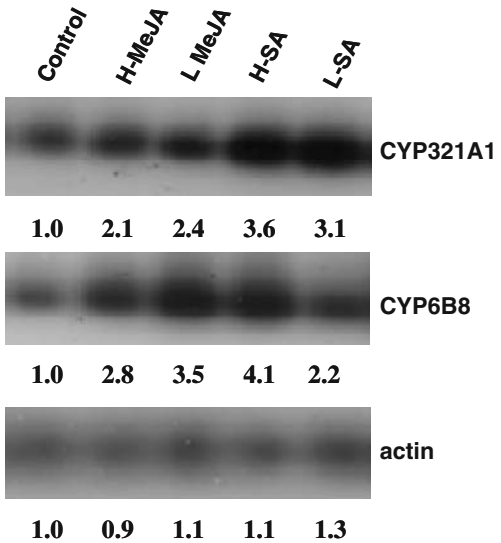

Fig. 5 P450 transcripts expressed in response to methyl jasmonate (MeJA) and salicylate (SA). Total *Helicoverpa zea* midgut RNA samples from the different diet groups (DMSO, 0.2% control solvent for all chemicals; H-MeJA, MeJA at 100 µg/g diet; L-MeJA, MeJA at 2.9 µg/g diet; H-SA, SA at 1 mg/g diet; L-SA, SA at 12 µg/g diet) were separately amplified by RT-PCR and analyzed as described in the Methods and Materials. The fold induction for each RT-PCR amplification after normalization to the constitutive actin transcript is shown below each lane

that bring about the detoxification of plant allelochemicals in the majority of cases.

In contrast with most plant chemicals, however, many mycotoxins, including aflatoxins, ochratoxin A, and zearalenone, are bioactivated by P450s or other oxidative enzymes (Dowd 1992; Ioannides 2000; Yiannikouris and Jouany 2002). The fungal toxin griseofulvin, synthesized by several *Penicillium* species, in fact appears to be bioactivated by P450s in *H. zea* (Dowd 1993). Producing toxins that are bioactivated by the principal Phase I metabolic enzymes used by the vast majority of animals may have been a key component of the adaptations of pathogenic fungi that allow them to defend their host resources against larger erstwhile consumers (Janzen 1977) (but see Sherratt et al. 2006). Plant signaling substances are well known to play distinct roles in mediating tritrophic interactions (Kessler and Baldwin 2002). In interactions between plants and insects, they initiate production of indirect defenses, i.e., volatile attractants that recruit predaceous natural enemies, and direct defenses, i.e., volatile repellents to herbivores (Heil 2004). In multitrophic interactions among plants, insects, and fungi, they may under some circumstances confer additional benefits to the plant by rendering insect consumers more vulnerable to fungal toxins. Whether this tritrophic interaction is a product of selection or is fortuitous is open to speculation.

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Bisorbicillinoids Produced by the Fungus *Trichoderma citrinoviride* Affect Feeding Preference of the Aphid *Schizaphis graminum*

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Abstract We report the effects of some bisorbicillinoids isolated from biomass of the fungus *Trichoderma citrinoviride* on settling and feeding preference of the aphid *Schizaphis graminum*. Purification of the fungal metabolites was carried out by a combination of column chromatography and thin-layer chromatography using direct and reverse phases. Chemical identification was performed by spectroscopic methods including nuclear magnetic resonance and mass spectrometry. The identified bisorbicillinoids appeared to be bislongiquinolide, its 16,17-dihydro derivative, trichodimerol, and dihydrotrichodimerol. A feeding preference test with alate morphs of *S. graminum* was used to identify the active fractions. Among the four bisorbicillinoids, dihydrotrichodimerol and bislongiquinolide influenced aphid feeding preference, restraining specimens from settling on leaves treated with metabolites. Taste neurons sensitive to these compounds, particularly to bislongiquinolide, were located on tarsi of the *S. graminum* alate morphs.

Keywords Aphids · *Schizaphis graminum* · *Trichoderma citrinoviride* · Bisorbicillinoids · Bislongiquinolide and 16,17-dihydrobislongiquinolide · Trichodimerol · Dihydrotrichodimerol · Feeding preference · Electrophysiological tests · Biocontrol

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Introduction

There has been growing interest in isolation of novel microbial metabolites for potential agricultural use (Tanaka and Ōmura 1993; Saxena and Pandey 2001). Microbial metabolites may reduce the selective pressure on crop pests due to the heavy and continuous use of synthetic agrochemicals and thus prevent the onset of resistant populations of plant pathogens and insects (Tanaka and Ōmura 1993). Furthermore, they are generally regarded as more environmentally friendly than their synthetic counterparts. The search for bioactive metabolites from microbes aims at the direct use of these compounds as agrochemicals as well as the discovery of new structural skeletons upon which to pattern synthesis of molecules with improved efficacy, stability, or biodegradability.

Fungi are an abundant source of secondary metabolites with various biological activities. In particular, fungal metabolites can affect different aspects of insect biology, such as survival, development, fecundity, and feeding

preference. The latter may have the ecological benefit of protecting fungal mycelium from mycophagous arthropods, such as springtails (Sabatini and Innocenti 2000). An eventual use of metabolites that are able to influence the feeding preference of insects in agriculture is conceivable. For instance, compounds that restrain aphids from feeding may reduce direct insect damage to crops and indirect damage due to infection with insect-transmitted viruses (Pickett et al. 1987). Data on such effects of fungal metabolites towards insect pests are limited (Wright et al. 1982; Mulè et al. 1992; Amiri et al. 1999; Ekesi et al. 2001; Ganassi et al. 2007). Ganassi et al. (2007) showed that some fungal isolates of the genus *Trichoderma* influenced the feeding preference of *Schizaphis graminum*, one of the most important pests of cereal crops, suggesting a potential antifeedant activity of these fungi towards aphids. The authors also showed that the biological activity of the fungal culture was from the water–methanol extractable of the fungal biomass, thus implying the presence of diffusible metabolites. Recently, two novel metabolites with potential antifeedant activity, citrantifidiene and citrantifidiol, were isolated from the culture extract of *Trichoderma citrinoviride* strain ITEM-4484 and characterized as a symmetrical disubstituted hexa-1,3-dienyl ester of acetic acid and a tetrasubstituted cyclohexane-1,3-diol, respectively (Evidente et al. 2008).

In this paper, we report the isolation and detection of four additional metabolites from strain ITEM-4484, all belonging to the bisorbicillinoids, a chemical family distinct from citrantifidiene and citrantifidiol. For two of them (trichodimerol and dihydrotrichodimerol), the genus *Trichoderma* is herein reported as a source for the first time. The effects on aphids of bisorbicillinoids from *T. citrinoviride* was studied by feeding preference tests and electrophysiological assays. These allowed us to characterize bislongiquinolide (**1**) and dihydrotrichodimerol (**4**) as two additional *Trichoderma* metabolites that influence aphid feeding preference, restraining them from settling on treated leaves.

Methods and Materials

Organisms

Fungus The fungal strain used was isolated from soil under *Abies* sp. in Tyrol (Austria). After re-isolation from a single germinated conidium, the strain was maintained in purity in the culture collection of the Institute of Sciences of Food Production, Bari, Italy, with the accession *T. citrinoviride* ITEM-4484. The strain was identified on the basis of morphological characters and by molecular techniques (sequence analysis of the internal transcribed spacer regions ITS-1 and ITS-2 of the nuclear rDNA, and a fragment of the translation elongation factor gene TEF-1α) as recently reported (Evidente et al. 2008).

Aphids The aphids used belonged to the species *S. graminum* (Rondani) (Hemiptera: Aphididae). The original *S. graminum* stock was collected in a cereal field located near Modena (Italy). Aphids were reared for several generations on wheat plants (*Triticum durum* Desf.) in a thermostatic chamber at 20°C under a 16:8 h light/dark cycle to induce parthenogenesis. Trials were carried out using alate adult morphs. Alate offspring were obtained in the laboratory by crowding.

Extraction and Purification of Fungal Metabolites Metabolites of *T. citrinoviride* ITEM 4484 were produced in a solid state fermentation as previously reported (Evidente et al. 2008). Briefly, the fungus was grown on autoclaved rice kernels brought to 45% moisture. Cultures were incubated at 25°C under a photoperiod of 12 h for 4 weeks and then oven-dried and finely ground. One kilogram of dried material was extracted with MeOH–H_2O (1% NaCl) mixture (55:45 *v*/*v*), defatted, and then extracted with CH_2Cl_2 as reported elsewhere (Evidente et al. 2008). The organic extracts were combined, dried (Na_2SO_4), and evaporated under reduced pressure, yielding a brown oil (15 g). The organic extracts (15 g) were fractionated by column chromatography (CC; eluent, $CHCl_3$-*i*-PrOH 85:15), and the fractions collected were analyzed by thin-layer chromatography (TLC) on silica gel (same solvent). Fractions showing the same chromatographic pattern were pooled yielding ten groups (A–L) of homogeneous fractions. The last fraction collected after fractions A–L was eluted with methanol.

A feeding preference test with alate morphs of the aphid *S. graminum* was used to identify active fractions in this and all subsequent purification steps. Residues of the B (1.58 g) and C (1.28 g) fraction groups were active in restraining aphids from settling on treated leaves (data not shown). The residue of fraction B (1.58 g) was purified by column chromatography (eluent, petroleum ether–Me_2CO 7:3), yielding ten groups (B1–B10) of homogeneous fractions. Only the residue of fraction B4 (94.7 mg) showed high activity and was purified further in two successive steps by preparative TLC on silica gel (eluent, $CHCl_3$-*i*-PrOH 9:1) and reversed-phase (eluent, EtOH–H_2O 6:4), yielding the most abundant active metabolite as a homogeneous solid. This was identified as dihydrotrichodimerol, (**1**, R_f 0.63 and 0.23, silica gel eluent, $CHCl_3$-*i*-PrOH 9:1, and reverse phase eluent, EtOH–H_2O 6:4, respectively, 15 mg). Purification by column chromatography (eluent, petroleum ether–Me_2CO

7:3) of the residue of fraction C (1.28 g) of the initial column gave nine groups of homogeneous fractions (C1–C9). The residues of fractions C2 (80.1 mg), C3 (431.6 mg), and C7 (54.6 mg) showed high activity. Purification of fraction C2 by preparative TLC (eluent, $CHCl_3$-*i*-PrOH 9:1) gave seven groups of homogeneous fractions (A1–G1). The residues of fractions D1 (15 mg) and G1 (8.8 mg) were active in feeding preference tests. Fraction D1 was purified by preparative TLC yielding citrantifidiene (Evidente et al. 2008), while fraction G1 (7.5 mg) by preparative TLC on reverse (eluent, EtOH–H_2O 6:4) yielded a homogeneous solid that was identified as trichodimerol (**2**, 4.6 mg R_f 0.4, reverse phase eluent, EtOH–H_2O 6:4). Purification of fraction C3 by a combination of column chromatography and TLC yielded citrantifidiol (Evidente et al. 2008). Finally, purification of fraction C7 by TLC on reverse phase (eluent, EtOH–H_2O 6:4) gave two homogeneous solids, the first yellow colored, and these were identified as bislongiquinolide (**3**, 7.5 mg, R_f 0.51 reverse phase eluent, EtOH–H_2O 6:4) and 16,17-dihydrobislongiquinolide (**4**, 12.0 mg, R_f 0.42 reverse phase eluent, EtOH–H_2O 6:4).

Identification Optical rotation was measured in $CHCl_3$ solution on a JASCO (Tokyo, Japan) P-1010 digital polarimeter; infrared (IR) and ultraviolet (UV) spectra were recorded as deposit glassy film and in MeCN solution, respectively, on a Perkin-Elmer Spectrum One Fourier transform-IR Spectrometer and a Perkin-Elmer Lambda 25 UV/Vis spectrophotometer (Norwalk, CT, USA). ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 600 (with a TCI cryoprobe™) and at 150 and 75 MHz, respectively, in $CDCl_3$ on Bruker spectrometers (Karlsruhe, Germany). Solvent was used as internal standard. Carbon multiplicities were determined by distortionless enhancement by polarization transfer (DEPT) spectra (Berger and Braun 2004). DEPT, correlation spectroscopy (COSY)-45, heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) experiments (Berger and Braun 2004) were performed using Bruker microprograms. Electrospray ionization (ESI) and high-resolution (HR) ESI mass spectrometry (MS) spectra were recorded on Waters Micromass Q-TOF Micro spectrometer (Milford, MS, USA), while electron ionization (EI)-MS were taken at 70 eV on a QP 5050 Shimadzu spectrometer (Kyoto, Japan). Analytical and preparative TLC were performed on silica gel (Kieselgel 60 F_{254}, 0.25 and 0.50 mm, respectively, Merck, Darmstadt, Germany) or reverse-phase (Whatman, KC18 F_{254}, 0.20 mm, Maidstone, UK) plates; the spots were visualized by exposure to UV light and/or by spraying first with 10% H_2SO_4 in methanol and then with 5% phosphomolybdic acid in ethanol, followed by heating at 110°C for 10 min. Column chromatography: silica gel (Kieselgel 60, 0.063–0.200 mm Merck, Darmstadt, Germany).

Dihydrotrichodimerol (**1**) $[\alpha]_D^{25}$: +58.0 (*c* 1.4); IR ν_{max} cm^{-1} 3,423, 1,719, 1,634, 1,614, 1,548; UV λ_{max} nm (log ε) 360 (4.61), 298 (4.56), 234 (4.25); ^1H and ^{13}C NMR spectra were identical to those previously reported (Liu et al. 2005); HR ESI MS (+) *m/z*: 537 $[M+K]^+$, 521.2136 [$C_{28}H_{34}O_8Na$, calcd. 521.2151] $[M+Na]^+$, 499 $[M+H]^+$; ESI MS (−) *m/z*, 497 $[M-H]^-$; EI-MS (rel. int) *m/z*: 498 $[M]^+$ (15) 480 $[M-H_2O]^+$ (2), 401 $[M-CH_3-CH=CH-CH_2-CH_2-CO]^+$ (4), 384 $[M-CH_3-CH=CH-CH_2-CH_2-CO-OH]^+$ (1), 95 $[CH_3-CH=CH-CH=CH-C=O]^+$ (100), 55 $[CH_3-CH=CH-CH_2]^+$ (99).

Trichodimerol (**2**) $[\alpha]_D^{25}$: −408 (*c* 0.1); IR ν_{max} cm^{-1} 3,394, 1,613, 1,587, 1,415, 1,378; UV λ_{max} nm (log ε): 363 (4.60), 308 (4.30), 241 (4.15); ^1H and ^{13}C NMR spectra were identical to those previously reported (Liu et al. 2005); ESI MS (+) *m/z*: 519 $[M+Na]^+$, 497 $[M+H]^+$.

Bislongiquinolide (**3**) $[\alpha]_D^{25}$: +130 (*c* 0.2); IR ν_{max} cm^{-1} 3,434, 1,757–1,720, 1,630 1,576; UV λ_{max} nm (log ε) 373 (4.06), 290 (4.21) 261 (4.19); ^1H NMR spectrum was identical to that previously reported by Shirota et al. (1997) and Sperry et al. (1998); ESI MS (+) *m/z*: 519 $[M+Na]^+$.

Dihydrobislongiquinolide (**4**) $[\alpha]_D^{25}$: +350 (*c* 0.1); IR ν_{max} cm^{-1} 3,380, 1,729, 1,628, 1,570, 1,447, 1,379; UV λ_{max} nm (log ε) 369 (4.45), 259 (4.58); ^1H and ^{13}C NMR spectra were identical to those previously reported (Shirota et al. 1997); ESI MS (+) *m/z*: 521 $[M+Na]^+$.

Behavioral Bioassays The biological activity of the raw organic extract of *T. citrinoviride*, of the chromatographic fractions obtained in the purification process (data not shown), and of pure bisorbicillinoids (**1**–**4**), was assessed by feeding preference tests on alate morphs of the aphid *S. graminum.*

Extract, chromatographic fractions, and pure compounds were solubilized in 5% methanol to perform feeding preference tests as previously described (Ganassi et al. 2007). The raw organic extract of the fungal culture was tested at a concentration corresponding to 500 mg of rice culture per ml of 5% aqueous methanol; dihydrotrichodimerol (**1**) at concentrations of 2.5, 1.25, and 0.625 mg ml^{-1}; trichodimerol (**2**) at 0.82 mg ml^{-1}; bislongiquinolide (**3**) at 0.68 and 0.34 mg ml^{-1}; and dihydrobislongiquinolide (**4**) at 0.71 mg ml^{-1}. A 5% methanolic solution was used as control. For the tests, excised wheat leaves about 2 cm in length were dipped in the different solutions for 10 s. Then, leaves were placed on wet filter paper in 12 cm Petri dishes. Each dish contained two leaves, one treated with test

solution and one dipped in control solution, arranged in parallel at a distance of 4 cm. Aphids were placed between the two leaves with a fine brush, and their position was recorded every hour for 8 h, starting from the initial access that began less than 1 h after leaf excision.

For each treatment, five alate morphs were tested separately, and six replicates were run. The entire test was performed three times with the organic extract and twice with chromatographic fractions (data not shown) and pure metabolites.

Statistical Analysis The raw data obtained from feeding preference tests were analyzed by the Generalized Linear Model (GLM) repeated measures (time) procedure and compared by using a test of within-subjects effects, with SPSS release 15.01 for Windows software (SPSS, Chicago, IL, USA). The differences between the means of the number of aphids per leaf in each of the experimental treatments and those of the number of aphids on related controls over time were analyzed and adjusted with Bonferroni test for the number of comparisons.

Electrophysiological Bioassays Alate and apterous morphs of *S. graminum* obtained from laboratory rearing on host plants were used. Different concentrations (2.48, 1.24, 0.62, and 0.31 mg ml^{-1} in a 100 mM NaCl aqueous solution with 5% methanol) of the bisorbicillinoids, trichodimerol, dihydrotrichodimerol, and bislongiquinolide were tested. Methanol was added to solubilize the test compounds. Although methanol is thought to damage the nerve cells, which then does not allow for long recording durations, and most likely influences impulse frequencies, it has the advantage of dissolving material obstructing the pores and helping to obtain a good contact (Maher and Thiery 2004). Electrophysiological responses from the mesothoracic distal tarsomere were recorded by combining different techniques previously used to study single chemosensory sensilla (Hodgson et al. 1955; den Otter and van der Starre 1967; Marion-Poll and van der Pers 1996; Solinas et al. 2001; Maher and Thiery 2004; Ganassi et al. 2007). The indifferent electrode, a glass micropipette (tip 4–5 μm diameter) filled with a ringer solution (Kaissling 1995), was inserted into the aphid prothorax. The recording electrode, a glass micropipette (tip 2 μm diameter) containing one of the test stimuli, was connected to a sensillum. Action potentials were preamplified, filtered, and recorded with commercially available electrophysiological equipment (Tasteprobe Type DTP-1, Syntech, Hilversum, The Netherlands).

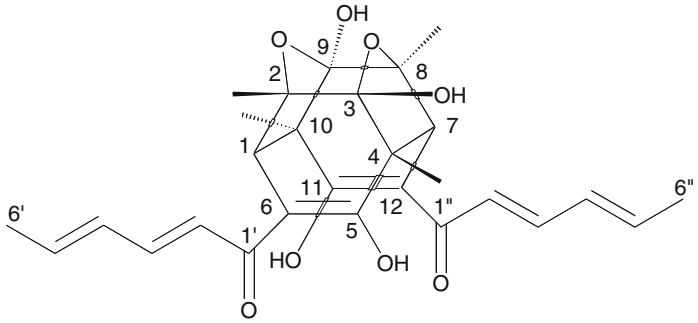
Test solutions were put into the micropipette 10 s before the experiment. Single-cell recordings were carried out at 22±2°C and 70±10% room humidity. Electrical activity was recorded for 1 s after stimulus onset, and 5 min was allowed to elapse between presentation of successive stimuli to the same sensillum. Test and control solutions were applied in a random series on the same sensillum. Action potentials (spikes) were stored on a magnetic tape (CditII, IEC II/Type II, High Bias 70 ms EQ, position chrome, Sony, Pontonx, sur l'Adour, France) by a double-channel recorder (Sony, TC-D5M) and successively analyzed with the AutoSpike™ 3.1 program (Syntech) on the basis of their amplitude, shape, and frequency.

Sensilla that failed to respond to the tested solutions were considered not functioning and were discarded (Crnjar and Prokopy 1982; Ganassi et al. 2007). Responses of the sensory cells were evaluated as spike frequency (spikes per second) during the first second of stimulation, 100 ms after stimulus onset. Firing frequencies were compared by means of the Student's *t* test.

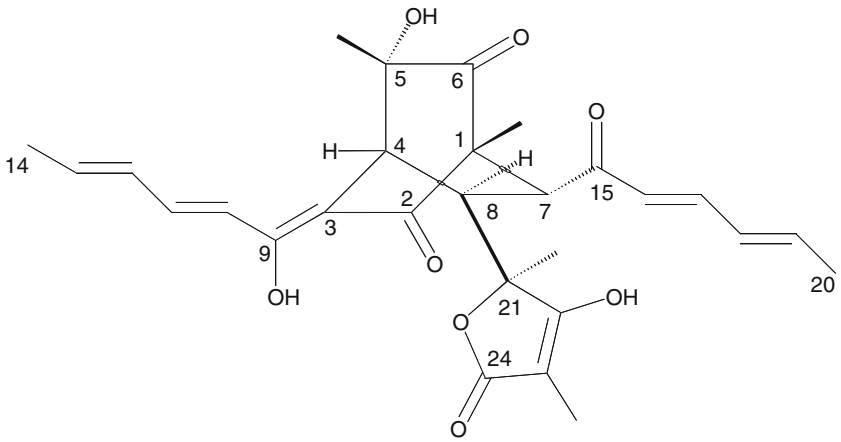
In order to verify the possible occurrence of olfactory responses, all compounds (25 μl of a 2.48 mg ml^{-1} solution) also were tested on antennae of living aphids. Stimuli were absorbed on a piece of filter paper (1 cm^2) inserted in a Pasteur pipette. Antennal responses were obtained by using a standard electroantennographic (EAG) technique (Ambrosi et al. 2001; De Cristofaro et al. 2004), and the green leaf volatile (*Z*)-3-hexen-1-ol (25 μl of a 1:10 *v/v* diluted solution in mineral oil) was used as an active control stimulus.

Results

The exhaustive extraction of a solid culture of *T. citrinoviride* (1 kg) yielded a crude organic extract that was active in restraining aphids from settling on treated leaves. This extract was purified by combined CC and TLC on direct and reverse phases and monitored by bioassays. This process led to the isolation of citrantifidiene and citrantifidiol, a symmetrical disubstituted hexa-1,3-dienyl ester of acetic acid, and a tetrasubstituted cyclohexane-1,3-diol, respectively, both with high activity towards aphids, and four additional metabolites as homogeneous solid compounds that did not crystallize. Preliminary ^{1}H and ^{13}C investigations showed that these metabolites were closely related and belonged to the family of bisorbicillinoids. Their structures were identified by using spectroscopic methods, essentially 1D (^{1}H and ^{13}C) 2D NMR (COSY, HSQC, HMBC) and EI and ESI MS techniques. The first metabolite was bislongiquinolide (**3**, Fig. 1, 7.5 mg kg^{-1}), based on its spectroscopic and physical properties. These were identical to those previously reported for this compound by Sperry et al. (1998), who originally characterized it from a strain of *Trichoderma longibrachiatum* isolated from *Haliclona* sp. from southwestern Indonesia. A second metabolite was identified as the 16,17-dihydroderivative of bislongiquinolide (**4**, Fig. 1, 12 mg kg^{-1}). The spectroscopic and physic properties were identical to those previously reported for **4** when first described by Shirota et al. (1997) as a metabolite



1 Dihydrotrichodimerol: 2',3'-dihydro
2 Trichodimerol



3 Bislongiquinolide
4 Dihydrobislongiquinolide: 16,17 dihydro

Fig. 1 Structures of dihydrotrichodimerol, trichodimerol, bislongiquinolide, and its 16,17-dihydroderivative (**1**–**4**)

of a *Trichoderma* sp. strain isolated from rice plant straw and named dihydrotrichotetronine. The third and the fourth metabolites were identified as dihydrotrichodimerol and trichodimerol (**1** and **2**, Fig. 1, 15 and 4.6 mg kg^{-1}, respectively). Their spectroscopic and physical properties were identical to those previously reported by Liu et al. (2005), who identified these compounds from a marine-derived strain of *Penicillium terrestre*.

Feeding Preference Tests on S. graminum Data obtained in tests carried out with wheat leaves treated with organic extract, with some of the chromatographic fractions from the purification process (data not shown), with pure metabolites, and with control leaves were analyzed with the GLM repeated measures procedure. This analysis determined if the interaction between the two conditions (treated and control) and the change over the time was statistically significant. The GLM analysis showed that the interaction between the number of aphids per leaf treated with organic extract of *T. citrinoviride* and the number of aphids per control leaf did not change over time; the Bonferroni adjustment for the number of comparisons revealed that the mean of the numbers of aphids counted on leaves dipped in organic extract was significantly smaller than the mean numbers of aphids counted on correspondent control leaves over time (Table 1).

GLM analysis of the data obtained in tests carried out with dihydrotrichodimerol (**1**) at the concentration of 2.5 mg ml^{-1} revealed no time × treatment interaction effects. The same analysis of the data obtained with a concentration of 1.25 mg ml^{-1} revealed time × treatment interaction effects, and also in this case the number of aphids counted on control leaves increased over the time of the trial. GLM analysis of the data obtained in tests carried out with a concentration of 0.625 mg ml^{-1} revealed no time × treatment interaction effects (Table 1). The Bonferroni test revealed that the average number of aphids on leaves treated with dihydrotrichodimerol at the concentrations of 2.5 and 1.25 mg ml^{-1} was significantly smaller than the number of aphids on correspondent control leaves, respectively, over time. The average number of aphids counted on leaves treated with dihydrotrichodimerol at a concentration of

Table 1 Effect of organic extract and pure metabolites of *T. citrinoviride* ITEM 4484 at different concentrations on feeding preference of *S. graminum*

Compounds	GLM **(time × treatment)**[a]		**Bonferroni test**[b]		
			Mean treatment	Mean control	
Organic extract	$F_{7,238}$=1.512	P>0.05	0.694±0.149	3.521±0.149	**
Dihydrotrichodimerol					
2.5 mg/ml	$F_{7,154}$=0.704	P>0.05	0.729±0.205	3.396±0.205	**
1.25 mg/ml	$F_{7,154}$=4.185	P<0.01	1.104±0.256	2.583±0.256	**
0.625 mg/ml	$F_{7,154}$=0.322	P>0.05	2.198±0.330	2.292±0.330	ns
Bislongiquinolide					
0.68 mg/ml	$F_{7,154}$=2.245	P<0.05	1.260±0.295	2.875±0.295	**
0.34 mg/ml	$F_{7,154}$=4.664	P<0.01	1.646±0.388	2.854±0.388	*
Trichodimerol					
0.825 mg/ml	$F_{7,154}$=3.645	P<0.01	2.417±0.246	2.208±0.246	ns
Dihydrobislongiquinolide					
0.71 mg/ml	$F_{7,154}$=0.294	P>0.05	1.833±0.343	2.375±0.343	ns

GLM values describe the effect of time on aphid feeding preferences

ns not significant

*P<0.05; **P<0.01

[a] Values of P>0.05 for GLM indicate that the interaction between the two conditions (treated and control) and the change over time was not statistically significant

[b] Differences between the means of the number of aphids per leaf in each of the experimental treatments and those of the number of aphids on related controls over time were analyzed and adjusted with Bonferroni test for the number of comparisons

0.625 mg ml^{-1} was not significantly different from the number of aphids counted on control over time (Table 1).

GLM analysis of the data obtained in tests carried out with trichodimerol (**2**) at a concentration of 0.825 mg ml^{-1} revealed time × treatment interaction effects, as the difference between treated and controls changed during the trial. The Bonferroni test revealed that the average number of aphids on leaves treated was not different from the number of aphids on correspondent controls over time (Table 1).

GLM analysis and Bonferroni test of the data obtained in tests carried with the pure metabolite bislongiquinolide (**3**) at a concentration of 0.68 mg ml^{-1} and at a concentration of 0.34 mg ml^{-1} showed a time × treatment interaction effect. Indeed, the number of aphids on control leaves increased over time of the trial (Table 1).

GLM analysis and Bonferroni test of the data obtained in tests carried out with dihydrobislongiquinolide (**4**) at a concentration of 0.71 mg ml^{-1} did not show any time × treatment interaction effects (Table 1).

Electrophysiological Bioassays EAG study showed that the tested bisorbicillinoids were not able to stimulate the antennal olfactory sensilla of either alate or apterous morphs of *S. graminum*. The antennae, on the contrary, were stimulated (>1.0 mV) by the control stimulus, the green leaf volatile (*Z*)-3-hexen-1-ol.

Electrophysiological bioassays performed on the meso-thoracic distal tarsomere revealed that 31% of the alate and 15% of the apterous morph sensilla contacted gave rise to spike activity. Through applications of the control solution (100 mM NaCl), different action potentials were recorded. Methanol (5%) added to the control solution evoked spike frequencies similar to those obtained with the control solution alone (Table 2).

Each sensillum elicited action potentials by more than two different cells, but only the two larger ones in alate (a= 1.2 mV; b=0.7 mV) and apterous morphs (A=0.8 mV; B= 0.5 mV) were considered (Table 2). Responses from cells that elicited action potentials of lower amplitude (<0.5 mV) were not analyzable. In addition, higher concentrations of the test solutions induced deformations in spike shape and amplitude, so that responses could not easily be evaluated; lower concentrations evoked spike frequencies similar to the control.

On stimulation with the tested bisorbicillinoids, significant increases in spike frequency over controls were recorded. Cell a and b of alate *S. graminum* showed highly significant concentration-dependent responses to dihydrotrichodimerol and bislongiquinolide, respectively.

Table 2 Spike frequency (spikes per s±SD) recorded from cells with different action potential amplitudes (*a*=1.2 mV; *b*=0.7 mV; *A*=0.8 mV; *B*=0.5 mV) of *S. graminum* (alate and apterous morphs) tarsal sensilla (*N*=5) on stimulation with different doses of dihydrotrichodimerol, trichodimerol, and bislongiquinolide (**1**, **2** and **3**) and NaCl aqueous solution (100 mM), alone or added with methanol (5%)

Stimulus	Alate sensory cell		Apterous sensory cell	
	a	*b*	*A*	*B*
NaCl 100 mM	6.2±1.5	5.8±2.2	7.4±2.6	11.8±4.4
NaCl 100 mM+methanol 5%	6.4±2.0	6.3±3.2	8.2±2.2	10.6±4.8
Dihydrotrichodimerol, 2.48 mg ml^{-1}	38.8±6.6**	8.4±5.8	6.4±2.4	12.4±3.8
Dihydrotrichodimerol, 1.24 mg ml^{-1}	34.2±7.4**	8.2±4.0	8.6±2.8	9.8±5.4
Dihydrotrichodimerol, 0.62 mg ml^{-1}	20.0±5.6*	7.6±2.8	7.4±3.0	10.8±4.6
Dihydrotrichodimerol, 0.31 mg ml^{-1}	8.2±4.6	7.6±4.6	7.2±2.8	12.8±4.8
Bislongiquinolide, 2.48 mg ml^{-1}	6.4±4.0	82.4±10.6***	6.8±4.6	20.8±3.8*
Bislongiquinolide, 1.24 mg ml^{-1}	7.2±3.8	72.6±12.8***	7.4±2.6	10.6±4.6
Bislongiquinolide, 0.62 mg ml^{-1}	8.6±4.6	42.4±8.4**	8.2±2.6	12.6±3.4
Bislongiquinolide, 0.31 mg ml^{-1}	7.4±3.0	12.4±7.6	7.8±3.0	11.4±4.8
Trichodimerol, 2.48 mg ml^{-1}	23.6±7.2*	8.4±6.6	7.4±4.2	13.2±5.0
Trichodimerol, 1.24 mg ml^{-1}	20.8±6.6*	7.4±5.8	8.4±2.6	12.8±3.4
Trichodimerol, 0.62 mg ml^{-1}	12.6±8.2	6.4±4.4	7.6±2.2	11.2±4.6
Trichodimerol, 0.31 mg ml^{-1}	9.6±6.4	9.4±5.8	7.8±2.8	11.0±5.2

In each experiment, responses by the two cells showing the higher spike amplitudes were analyzed

Referred to the same column, Student's *t* test shows significant differences

*$P<0.05$; **$P<0.01$; ***$P<0.001$

Cell a was also stimulated by the two higher concentrations of trichodimerol. Sensory cells of the apterae morphs were not sensitive to the tested compounds, with the exception of a weak response of cell b to bislongiquinolide (Table 2).

Discussion

The four metabolites isolated from *T. citrinoviride* are related compounds belonging to the subgroup of dimeric methylated hexaketides, named bisorbicillinoids. This family includes the parent compound sorbicillinol and more than 30 monomeric and dimeric vertinoids isolated from *Verticillium intertextum*, *Penicilllium*, and *Trichoderma* spp. with different origins (Maskey et al. 2005). Many studies have been carried out on their isolation and chemical characterization (Trifonov et al. 1983, 1986; Gao et al. 1995; Andrade et al. 1997; Shirota et al. 1997; Abe et al. 1998, 1999; Sperry et al. 1998; Maskey et al. 2005), their biosynthesis (Sperry et al. 1998; Abe et al. 2002), as well as their synthesis and biomimetic synthesis (Nicolaou et al. 1999, 2000; Pitsinos et al. 2008). In particular, bislongiquinolide (**3**) was previously isolated, together with an epoxysorbicillinoid, a new vertinoid pigment, from a *T. longibrachiatum* strain isolated from the marine sponge *Haliclona* sp. (Sperry et al. 1998). In the same paper, the authors established that **3** and trichotetronine, which, consequently, was re-named bislongiquinolide, were identical. Trichotetronine had been previously described as a metabolite of a *Trichoderma* sp.; likewise, the 24,25-dihydroderivative named dihydrotrichotetronine (Shirota et al. 1997) corresponds to the dihydrobislongiquinolide isolated in the present paper (**4**). Interestingly, the species *T. longibrachiatum* and *T. citrinoviride* are closely related, as they both belong to the *Trichoderma* section *Longibrachiatum* and to the same phylogenetic clade (Bissett 1984, 1991; Samuels et al. 1998). Unfortunately, the third *Trichoderma* strain thus far known to produce bisorbicillinoids was not identified to species level (Shirota et al. 1997). It seems that the production of bisorbicillinoids is diffused among, if not restricted to, the species of the *Trichoderma* section *Longibrachiatum*. Should this observation be supported by further studies, it would be of importance for both the systematics of *Trichoderma* based on profiles of secondary metabolites (chemotaxonomy) and for screening of isolates producing new compounds that belong to this class of bioactive compounds. Both *T. longibrachiatum* and *T. citrinoviride* are soil inhabitants, especially abundant in natural and forest soils. Therefore, the production of fungal metabolites, which influence feeding preference of aphids, might be ecologically related to self-defense mechanisms against soil animals that eat

fungi. A definitive stereostructure was assigned to **3**, and a new skeleton numbering was assigned to these metabolites in agreement to that adopted for the dimeric bisorbicillinoid subgroup (Sperry et al. 1998). Trichodimerol and dihydrotrichodimerol previously were isolated, together with tetrahydrotrichodimerol, from a marine-derived strain of *P. terrestre*. Their cytotoxic effects were evaluated by the MTT method (Mosmann 1983) in P338 and A-459 cell lines using VP16 as a positive control (Liu et al. 2005). Not surprisingly, this diverse array of structurally related natural products has a broad range of biological activities. For instance, dihydrotrichodimerol (**1**) specifically activated peroxisome proliferator-activated receptor γ without affecting peroxisome proliferator-activated receptors α and δ. On the other hand, compounds **1** and **2** suppressed the production of tumor necrosis factor-α and nitric oxide in LPS-stimulated RAW264.7 cells to a similar extent (Lee et al. 2005)

Bisvertinolone, isolated from *Acremonium strictum*, is a novel antifungal agent that functions via inhibition of β-(1,6)-glucan biosynthesis (Nicolaou et al. 2000). In addition, bisorbicillinol, bisorbibutenolide, bisorbicillinoide, and birsorbibetanone exhibit antioxidant properties (Nicolaou et al. 2000).

The activity of influencing aphid feeding, restraining them from settling on treated leaves of bislongiquinolide and dihydrotrichodimerol, is reported herein from behavioral and electrophysiological tests, for the first time. These findings are in agreement with results previously observed by using powdered rice cultures of the fungus *T. citrinoviride* in a test with *S. graminum* (Ganassi et al. 2007).

EAG study showed that the tested compounds, however, were not able to stimulate the olfactory sensilla of *S. graminum*. In a previous paper, we showed that mixtures of metabolites of some species of *Trichoderma* are detected by tarsal sensory neurons (Ganassi et al. 2007). Results of single-cell recordings reported here indicate that the structures involved in the perception of bisorbicillinoids are taste cells located on the aphid tarsomeres. Single-cell responses to bislongiquinolide (cell b of the alate morphs) and dihydrotrichodimerol (cell a of the alate morphs) are dose-dependent and consistent with behavioral results. The biological meaning of the response of the cell a to trichodimerol is not clear. This compound did not elicit any activity in behavioral tests, and sensory responses could be due to its chemical structure, closely similar to that one of dihydrotrichodimerol. It has been argued that the different sensitivity of the two morphs of *S. graminum* might be due to the different distribution and number of adequate gustatory receptors (Ganassi et al. 2007). This hypothesis is largely supported both by the lower percentage of taste cells that evoke spike activity in apterous morphs and by the lack of responses to the tested compounds.

It has been reported that *T. citrinoviride* can cause infections in immunosuppressed humans (Kuhls et al. 1999). In practice, this may prevent the use of viable cultures of the fungus but not of its metabolites capable of influencing aphid feeding preference.

The findings reported in this paper show that the metabolites of the fungus *T. citrinoviride*, namely, bislongiquinolide and dihydrotrichodimerol, as well as citroantifidiene and citroantifidiol (Evidente et al. 2008) that have been previously isolated from the same source and belong to other classes of natural compounds, have a potential for the development of new agrochemicals for control of the aphid *S. graminum*.

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Identification of Host Attractants for the Ethiopian Fruit Fly, *Dacus ciliatus* Loew

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Abstract The Ethiopian fruit fly, *Dacus ciliatus*, is an oligophagous pest of cucurbit crops, particularly melons, cucumbers, and marrows (summer squash). The present study aimed to identify host attractants for *D. ciliatus* and was guided by a behavioral bioassay and an electrophysiological assay. We tested volatile compounds from the fruits of a host plant, ripe and unripe Galia melon, *Cucumis melo* var. *reticulates*. Both sexes were attracted to melon volatiles. Those of ripe melon were preferred. Gas chromatography-electroantennographic detection analysis of the behaviorally active ripe melon volatiles consistently showed that 14 compounds elicited similar antennal responses from both sexes. Twelve compounds were identified by gas chromatography-mass spectrometry (GC-MS) using GC-MS libraries, retention indices (RI), and authentic standards. The electrophysiological activities of the compounds that were present at sufficient levels for identification, benzyl acetate, hexanyl acetate, (*Z*)-3-hexenyl acetate, (*Z*)-3-octenyl acetate, octanyl acetate, (*Z*)-3-decenyl acetate, and (*E*)-β-farnesene, were evaluated at six different dosage levels by using electroantennography (EAG). Benzyl and hexanyl acetates elicited dose responses only in males, while other tested compounds elicited dose responses in both sexes. The strongest responses were observed for doses between 100 ng and 10 μg. The dose response, in terms of attractiveness to synthetic compounds within the active range (as determined by EAG), also was evaluated in the behavioral bioassay. Synthetic acetates were attractive to both sexes when tested individually. Significant attraction was observed when individual compounds were applied in the bioassay arena at doses of 0.5–1 μg/dispenser. Blends of compounds in equal proportions also were attractive to the insects. The most attractive blend was a mixture of four or five identified acetates. The addition of an equal proportion of (*E*)-β-farnesene to this mixture had a deterrent effect.

Keywords Attractants · *Cucumis melo* · EAG · Fruit fly · GC-EAD · Plant volatiles · Diptera · Tephritidae

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Introduction

Fruit flies (Diptera: Tephritidae) are among the most important quarantine pests worldwide. Among these, the Ethiopian fruit fly, *Dacus ciliatus* Loew., is a serious pest of cucurbits in many countries, such as Egypt (Azab et al. 1970), South Africa (Hancock 1989), the island of Reunion, and other islands in the Indian Ocean (Dehecq 1995). This fly is classified as an A1 quarantine pest by the European and Mediterranean Plant Protection Organization (2008), requiring efficient monitoring and detection systems to reduce the probability of accidental introductions and export. Detection systems usually are based on strong olfactory attractants, which ideally are species-specific. In addition, lure-and-kill methods based on efficient attraction systems have proven valuable in the control of fruit flies (Aluja 1996; Hendrichs 1996; Koyama et al. 2004).

Little information is available regarding the chemical ecology of *D. ciliatus*, and no attractants have been identified for this species. There are several types of olfactory attractants for fruit flies: plant kairomones, parapheromones (that may include volatiles derived from host or nonhost plant species, such as trimedlure for *Ceratitis capitata* and methyl eugenol for many *Bactrocera* species),

food lures (that are usually protein hydrolyzate derivatives), and sex pheromones (Metcalf 1990; Jang and Light 1996). The attraction of *D. ciliatus* to some known parapheromones and essential oils was investigated several years ago by Qureshi et al. (1986). In that study, none of the investigated volatiles (e.g., Trimedlure, methyl eugenol, cue-lure, grapefruit oil, lemon oil, angelica seed oil, rose oil, etc.) attracted *D. ciliatus*. In a recent study performed in insect-proof screen cages in our laboratory, we confirmed the results of Qureshi et al. (1986), specifically regarding the lack of attraction of *D. ciliatus* to cue-lure, methyl eugenol, raspberry ketone, angelica seed oil, and Trimedlure (D. Nestel, unpublished results). We also observed low attraction to ammonia-releasing sources, such as BioLure, Buminal, etc. (D. Nestel, unpublished results).

Host and nonhost plants are the focal point of all fruit fly activities, such as feeding, lekking, mating, and egg-laying. Most of these activities are mediated by semiochemicals (Tan 2000). In particular, the attraction of fruit flies to their hosts is usually guided by volatile phytochemicals (Aluja and Mangan 2008), especially in species that exhibit host specificity such as oligophagous *D. ciliatus*. Indeed, a preliminary wind tunnel bioassay revealed that *D. ciliatus* is attracted to pieces of cucurbit fruits (e.g., melon and cucumber; D. Nestel, unpublished results).

Isolation and identification of specific attractants from fruit often is difficult due to blend complexity, which changes in composition during ripening. Rapid screening of multiple compounds of interest and the analysis of complex blends have been facilitated by coupled gas chromatography-electroantennogram detection (GC-EAD), which has been used successfully to identify attractants for a number of fruit fly species from several plant sources (Cosse et al. 1995; Zhang et al. 1999; Nojima et al. 2003a, b; Siderhurst and Jang 2006). The objective of the present study was to identify plant-derived attractants of *D. ciliatus* based on a behavioral bioassay coupled with gas chromatography-electroantennogram detection. Since the screening of volatiles by using a wind tunnel is a time-consuming process and field tests are problematic for quarantine pests, a cage choice bioassay was designed especially for this study.

Methods and Materials

Insect Culture Adult flies were obtained from a culture maintained at 27°C under strict quarantine conditions by the Plant Protection and Inspection Services of the Agricultural Research Organization (Israel Ministry of Agriculture), located in Bet Dagan, Israel. The culture was initiated 10 years ago from a wild population from the Arava valley. The culture is periodically refreshed with wild material. Larvae were reared on an artificial diet, as described in Nestel et al. (2004). Adults were provided with water and a mixture of sugar and yeast hydrolyzate. Sexually mature male and female flies, 10 to 16 days old, were selected prior to each experiment.

Fruits Ripe and unripe highly aromatic Galia melons, *Cucumis melo* var. *reticulates*, grown in open fields under commercial conditions were used. Melons were picked directly from the field and preserved for 3–4 h under cold conditions (~4°C) until volatile collection.

Collection of Volatile Compounds Host and nonhost fruits (ripe or unripe) were placed into jars of different sizes (0.5–5 l) with purified air passing through activated charcoal into the jar. Aerations were run for 48 h using a tube (3 cm×6 mm ID) of Super Q (Alltech Associates, Deerfield, IL, USA) held in place with glass wool plugs. The collected materials were extracted with 3 ml *n*-hexane (HPLC grade, Bio-Lab Israel, Jerusalem) and stored at 18°C for further assays.

Behavioral Bioassay Fly preferences for host volatiles were tested in a two-choice bioassay in a still-air arena. Plexiglas cages (40×40×30 cm^3) served as bioassay arena units. To avoid saturation with volatiles, the cages had two screened windows, each 10 cm wide. Experimental cages were located in a ventilated room, with a continuous interchange of air. Four traps, each consisting of a 20 ml glass vial plugged with a stainless steel funnel, were set in the corners of the cage floor. Two identical baited traps (treatment) and two control traps were set in opposing corners of each cage. To reduce the effects of directionality, baited and control traps were located within each replicate following a randomization protocol. Each bait (50–100 µl) (e.g., volatile preparations, different doses of individual compounds, or blends) and an appropriate control (*n*-hexane) were absorbed onto cotton wool wicks (1.9×1 cm ID). Bioassays consisted of releasing ten females or ten males in the center of a cage at around 1700 hours. Flies entering traps or remaining outside (alive or dead) were counted after 20 h. Each preparation was tested at least five times, usually simultaneously in different bioassay units situated side by side and exposed to the same illumination and environmental conditions. Initially, observations were made at 1 h intervals for the first 7 h and then again overnight. Data are reported as the percentage of flies entering either the treatment or the control traps overnight. The percentage of insects attracted to the tested preparation and to control treatment was calculated at each time interval by counting the cumulative number of flies in each kind of trap and dividing that number by the total number of trapped flies. Usually, for each replicate, seven to ten flies entered the traps after 20 h.

Identification of Antennae-Active Fruit Volatiles Electroantennographic responses to fruit volatiles were recorded with a Thermo Finnigan (Chrom-Card Trace-Focus GC, Milano, Italy) gas chromatograph coupled to a Syntech (IDAC-232, Hilversum, the Netherlands) electroantennogram detector system. The GC was equipped with a ZB-5 column (30× 0.25 mm ID×0.25 μm film thickness, Phenomenex (Torrance, CA, USA), with helium as the carrier gas at 2.0 ml/min and a make-up gas of 2 ml/min, which was added before the splitter. The injector, in splitless mode, and flame ionization detector (GC-FID) were kept at 250°C and 270°C, respectively. The oven was kept at 60°C for 2 min, and the temperature was then raised by 10°C min^{-1} to a final temperature of 230°C, which was maintained for 10 min.

For electroantenno-detection, the insect head was cut off, and a reference electrode was inserted into its base with a glass capillary tube filled with KCl (0.1 N). The distal end of the antenna was inserted into the tip of the recording glass capillary electrode. Antennae from both sexes were tested. Each antennal preparation was used only once. Humidified air (0.8 l/min) was used to carry effluent from the EAD transfer line to the antennal preparation. The signals generated by the EAD and FID were passed through a Syntech IDAC-232 high-impedance amplifier and analyzed with Syntech GC-EAD2000 software version 1.00. FID peaks that elicited EAD responses in at least seven runs were marked for identification by GC-mass spectrometry (GC-MS).

Electron Impact MS (70 eV) was conducted with an Agilent 6890 N GC coupled to an Agilent 5973 N Mass Selective Detector by a RTX-5SilMS (30×0.25 mm ID× 0.25 μm film thickness; Restek, Bellefonte, PA, USA) capillary column. Scanned masses ranged from 40 to 400 amu. The oven temperature was held at 50°C for 10 min, then heated at 15°C min^{-1} to 230°C, and kept at this temperature for 10 min. The injector was kept at 220°C in the splitless mode with helium (1.5 ml min^{-1}) as the carrier. Identification was conducted according to calculated Kovat's indices of the EAD runs, the mass spectra of EAD-active compounds that had been matched with MS libraries (NIST 5, Wiley 7 and Adams), literary Kovat's indices, and injections of authentic samples from synthesis or commercial sources as indicated below.

GC Analyses were conducted by using an Agilent 6890 GC equipped with a DB-23 30 m×0.25 mm ID×0.25 μm film-thickness polar column (J&W Scientific, Folsom, CA, USA) and a nonpolar HP-5 (J&W Scientific) capillary column, used in the splitless mode. The polar column was initially at 50°C for 3 min; the temperature was increased at a rate of 5° C min^{-1} to 220°C and was maintained at 220°C for 10 min. The nonpolar column was held at 50°C for 10 min, heated at 15°C min^{-1} to 230°C, and maintained at 230°C for 10 min. The injector was held at 230°C in the splitless mode with helium (1.5 ml min^{-1}) as the carrier. Pentadecanyl acetate was used as a standard for quantification.

The positions of the double bonds in hexenyl, octenyl, and decenyl acetates were determined by the microchemical reaction of dimethyl disulfide adducts according to the accepted procedure (Dunkelblum et al. 1985), followed by GC-MS analysis of the adduct products, as described above. The position of the double bond in dodecenyl acetate was not identified in the same way, since the amount of material available for reaction with dimethyl disulfide was too small. However, the double bond in this compound is most probably located at carbon 3, based on the results of our injections of seven different dodecenyl acetate standards, containing a double bond on carbon 3, 5, 7, 9, 10, or 11. The geometries of the double bonds in hexenyl, octenyl, decenyl, and dodecenyl acetates were determined by comparing the Rt values of corresponding standards with the natural sample in the polar column.

Chemicals Chemicals obtained from commercial sources were used without further purification: (*Z*)-3-hexenol, *n*-hexanol, *n*-octanol (Fluka, Buchs SG, Switzerland), (*E*)-3-decenylacetate and (*Z*)-3-decenyl acetate (Pherobank, Wageningen, The Netherlands), benzyl alcohol and (*Z*)-3-octenol (Sigma-Aldrich, Rehovot, Israel), (*E*)-β-farnesene (Bedoukian, Danbury, CT, USA), β-caryophyllene (Fluka, Buchs, Germany), (*Z*)-3-dodecenyl acetate (old sample from unknown supplier), pyridine, and acetic anhydride (Aldrich, Steinheim, Germany). Alcohols were converted into their acetates by overnight reaction with acetic anhydride in pyridine, which yielded the appropriate acetates (90–99% yield). Liquid flash chromatography was performed by using silica gel 60 (70–230 mesh) and pentane, with increasing amounts (1–5%) of ether used as eluting solvents.

Antennal Response to Different Concentrations of Active Volatiles The receptivity of the antennae of both sexes of *D. ciliatus* to serially diluted solutions of synthetic chemicals was investigated with electroantennography (EAG). For each tested compound, a dilution series was prepared starting with a 1:1 (*w*/*w*) dilution in hexane (AR-grade) followed by further 1:10 dilutions to yield the following concentrations: 100 μg/μl, 10 μg, 1 μg, 100 ng, 10 ng, and 1 ng per 1 μl solution. Antennal preparations for the EAG analysis were made as described in the previous section. We used the same antenna to test all of the concentrations of a single compound. Each compound was tested on eight antennae, using one antenna per fly. To prepare the tested solutions for EAG, a standard aliquot (1 μl) of each test dilution was pipetted onto a piece of filter paper (Whatman No.1), exposed to air for 20 s to allow the solvent to evaporate

and then inserted into a glass Pasteur pipette. A stimulus flow controller (CS-05; Syntech) was used to generate a 0.6-s stimulus at 1–2 min intervals, with a flow rate of 1.5 l/min. The signals generated by the antenna were passed through a high-impedance amplifier (IDAC-232, Hilversum, the Netherlands) and displayed on a monitor. Syntech software was used to process EAG signals.

Statistical Analysis Differences in the proportion of flies selecting the tested compound or the control in the behavioral bioassay were analyzed with the Wilcoxon signed rank test. Inferences regarding the response of antennae to different concentrations of tested compounds in EAG were analyzed by using a repeated measures model with the General Linear Model (Statgraph Plus, 2000, Manugistics, Inc. Rockville, MA, USA). Average antennal responses were separated using the least significant difference (LSD) method (α=0.05; Statgraph Plus, 2000).

Results

Both sexes were attracted to volatile compounds collected from ripe melon (Wilcoxon signed rank test, Z=−2.11, P<0.034, df=7 for females; Z=−2.66, P<0.008, df=8 for males; Fig. 1). Insects were not attracted to volatiles from unripe melon. In the choice bioassay, both male and female flies were able to discriminate between volatile compounds collected from ripe melons and volatile compounds from unripe melons and preferred the former (Wilcoxon signed rank test, Z=−2.8, P<0.005, df=9 for females; Z=−2.8, P<0.005, df=9 for males; Fig. 1).

GC-EAD analysis of headspace volatiles from ripe Galia melon revealed about 14 compounds that consistently elicited electrophysiological responses from the antennae of both sexes of *D. ciliatus* (see EAD trace in Fig. 2). Compounds that elicited EAD responses were identified by GC-MS and GC analysis on nonpolar and polar capillary columns. The identification process was completed by subsequent injections of authentic standards to the two different polarity GC columns, except for isopentyl hexanoate, germacrene D, and cadinene (unknown isomer). The mass spectra and retention indices (RI) of these compounds are identical with those reported in the literature (El-Sayed, 2008; NIST 5 MS library; and references therein). Among the 14 active peaks associated with the elicitation of EAD responses, 12 compounds were identified: (*Z*)-3-hexenyl acetate, hexanyl acetate, benzyl acetate, (*Z*)-3-octenyl acetate, octanyl acetate, isopentyl hexanoate, (*Z*)-3-decenyl acetate (and some (*E*)-3-decenyl acetate), β-caryophyllene, (*E*)-β-farnesene, germacrene D, cadinene (unknown isomer), and (*Z*)-3-dodecenyl acetate (Table 1). Among the EAD-active ripe melon volatiles, hexanyl acetate and (*Z*)-3-hexenyl acetate had the most abundant peaks. The identified acetates were not found among the volatiles collected from unripe melons.

The sensitivity of fly antennae to increasing doses of individual compounds was analyzed by using EAG. As shown in Fig. 3, the responses of both male and female antennae to most compounds were dose-dependent (P<0.05). For both female and male insects, the optimal responses to most of the compounds occurs around 1 μg quantities. For some of the compounds, the level of the response differed between sexes. Males were more sensitive to hexanyl acetate (F=14.9, df=1.48, P<0.01), benzyl acetate (F=6.17, df=1.42, P<0.01), and *E*-(β)-farnesene (F=11.1, df=1.42, P<0.01) but less sensitive to (*Z*)-3-hexenyl acetate (F=14.1, df=1.42, P<0.01). Antennal responses to octanyl acetate and (*E*) and (*Z*)-3-decenyl acetate were not sex-dependent (F=0.82; F=0.46; F=1.97, respectively, P>0.05). For the response to (*Z*)-3-octenyl acetate, we found a significant interaction between sex and dosage (F=3.03; df=5; 48, P<0.05).

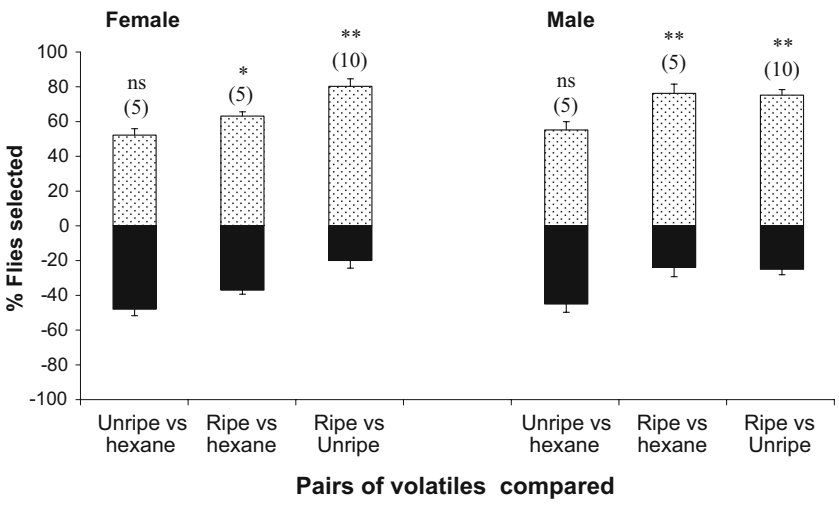

Fig. 1 *D. ciliatus* response to volatiles from ripe and unripe melons in an arena bioassay. Results are average percentages (±SE) of total flies trapped using each lure; *N*=number of cages tested for each set-up. *Dotted bars* represent responses to tested volatiles and *solid black bars* represent responses to the control (hexane or volatiles of unripe melon). **P*<0.05 Wilcoxon signed rank test, ***P*<0.01 Wilcoxon signed rank test, *ns* not significant

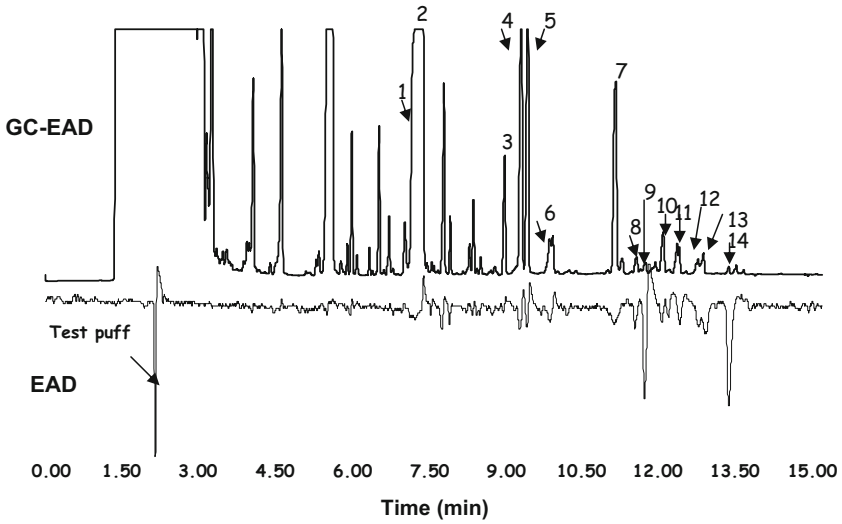

Fig. 2 GC-EAD profile of the ripe melon volatile extracts applied to the antennae of female *D. ciliatus*. The numbers represent peaks with EAD responses

The attractiveness of different doses of the identified EAD-active compounds was tested in a two-choice behavioral bioassay (Tables 2 and 3). In addition, 0.5 μg mixtures of active volatiles (increasing in compound content complexity) also were tested in the behavioral bioassay (Fig. 4). The results of the choice bioassay revealed that all of the acetates attracted flies of both sexes. For most compounds, significant attraction was observed when compounds were

Table 1 Volatile compounds from ripe melon fruits that elicited ead responses in the antennae of *D. ciliatus*

Peak #	Compound	Rt (min) EAD	RI[c]	RI[d]	EAD (mV)[a]		Amount (ng/μl)*	Prev. ref[b]
					Male	Female		
1	*Z*3-6:Ac	6.91	1,005	1,298	0.36±0.07	0.17±0.05	45	
2	6:Ac	7.22	1,014	1,278	0.69±0.06	0.53±0.09	153	Al; Cc; Bd
3	Benzyl acetate	8.83	1,169	1,720	0.44±0.06	0.42±0.06	15	
4	*Z*3-8:Ac	9.14	1,196	1,507	0.44±0.00	0.42±0.04	23	
5	8:Ac	9.27	1,209	1,488	0.63±0.04	0.73±0.09	20	
6	Isopentyl hexanoate	9.69	1,253		0.31±0.03	0.31±0.04	0.4	
7	*Z*3-10:Ac+minute amount of *E*3-10:Ac	10.90	1,389	(*Z*) – 1,720, (E) – 1,709	0.35±0.05	0.26±0.03	(*Z*)-15, (E)-0.5	
8a	N/A	11.35	1,438		0.57±0.005	0.40±0.04	Trace	
8b	B-Caryophyllene	11.38	1,442	1,627			1	Cc; Bt; Rp
9a	N/A	11.51	1,451		1.33±0.23	1.02±0.07	Trace	
9b	*E*-β-Farnesene	11.55	1,456	1,639			0.6	Cc; Bt; Bd
10	Germacrene D	11.87	1,503	N/A	0.44±0.08	0.18±0.06	3	
11	Cadinene (unknown isomer)	12.16	1,535	N/A	0.41±0.05	0.18±0.06	1.5	
12a	N/A	12.51	1,576	N/A	0.44±0.09	0.15±0.04	Trace	
12b	N/A	12.55	1,581	N/A			0.3	
13a	*Z*3 -12:Ac	12.59	1,587	1,940	0.43±0.07	0.15±0.06	0.5	
13b	N/A	12.66	1,602	N/A			2	
14	N/A	13.14	1,667	N/A	1.37±0.09	0.67±0.04	0.5	

[a] EAD values reflect the averages of five female and six male antennae depolarization records

[b] Compounds eliciting EAG responses in, or attracting other tephritids. The affected species are listed (*Al*, *A. ludens*; *Cc*, *C. capitata*; *Bd*, *B. dorsalis*; *Bt*, *Bactrocera tryoni*; *Rp*, *Rhagoletis pomonella*) (Cosse et al., 1995; Hull & Cribb, 2001; Hwang et al., 2002; Light et al., 1988; Malo et al., 2005; Nojima et al., 2003a; Siderhurst & Jang, 2006). Peaks marked by a and b are sequential with no base-line separation, therefore it is hard to determine which of them elicited the EAD-response. Pentadecanyl acetate was used as an external standard for quantification by FID.

[c] Rtx-5SilMS, Restek

[d] DB-23, J&W Scientific

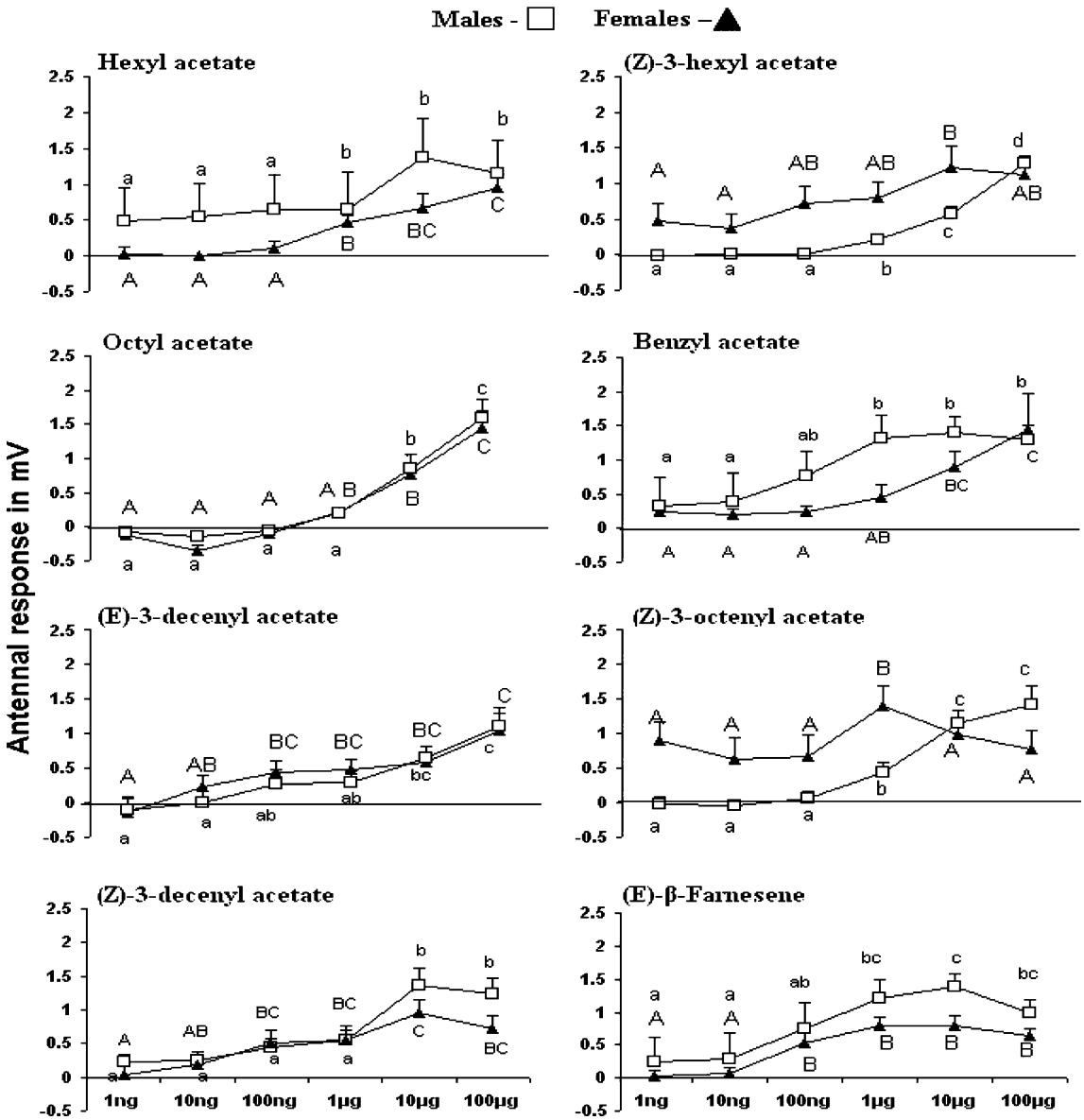

Fig. 3 EAG dose responses of female and male antennae of *D. ciliatus* to synthetic components of melon volatile blends. Results are average responses of at least eight antennae (expressed in mV). The response to hexane (blank) was subtracted from the data. Different letters (*capital letters* for females and *lower-case letters* for males) indicate significant differences in responses to ascending doses. GLM repeated measure following LSD analyses were conducted ($P<0.05$)

applied at doses around 0.5–1 µg. However, (*E*)-β-farnesene was slightly attractive (61%) to males (Wilcoxon signed rank test, $Z=-2.197$; $df=9$, $P=0.028$) but unattractive to females. Moreover, at some doses, it was even repellent.

Among the simple blends, the most effective attractant was a 1:1 mixture of octanyl acetate and (*Z*)-3-octenyl acetate (blend A; Fig. 4). Addition of (*E*)-β-farnesene to this mixture caused a decrease in attractiveness (blend C; Fig. 4). A blend of (*Z*/*E*)-3-decenyl acetate and hexanyl acetate (blend D) was even less attractive (Fig. 4). Overall, the most attractive blend for both sexes was a mixture of six acetates (blend G; Fig. 4). Benzyl acetate and (*E*)-β-farnesene were excluded from this mixture since, as mentioned above, the latter causes a decrease in attractiveness, especially to females, while the former was not that attractive to females in the dose–response behavioral bioassay (Table 3).

Discussion

Similar to many fruit flies, *D. ciliatus* was attracted to volatile compounds released by host fruit. In particular, volatile compounds from a climacteric melon variety (*C. melo* L. cv. *reticulates* group) were attractive to both sexes.

Table 2 Dose-dependent attraction of *D. ciliatus* females to 8 components of melon fruit volatiles

Amount (μg)	Benzyl acetate	Hexanyl acetate	(*Z*)-3-hexenyl acetate	Octanyl acetate	(*Z*)-3-octenyl acetate	(*E*)-3-decenyl acetate	(*Z*)-3-decenyl acetate	(*E*)-β-farnesene
0.125	57±7.5 (5)	66±8.2 (5)	54±6.0 (5)	49 ± 14.2 (5)	44 ± 4.6 (5)	56±5.7 (5)	55±4.8 (10)	34 ± 7.5 (5)#
0.25	NT	NT	59±7.0 (5)	60 ± 7.9 (5)	45 ± 3.7 (5)	60 ± 11.1 (5)	61 ± 6.7 (5)	58 ± 5.7 (10)
0.5	48±5.2 (13)	54±4.5 (18)	68±7.0 (13)*	70 ± 6.5 (5)#	67 ± 6.5 (5)#	54±5.2 (10)	88±5.6 (5)*	36 ± 4.1 (5)#
1	71±11.5 (5)#	51±8.3 (5)	54±6.7 (10)	71 ± 3.6 (10)**	75 ± 4.2 (11)**	72±3.5 (10)**	64 ± 7.0 (5)	47±9.1 (10)
1.5	59±4.6 (10)#	NT	NT	55 ± 6.5 (5)	65 ± 8.1 (5)	NT	78±10.8 (5)#	NT
2	NT	66±6.2 (10)#	NT	NT	NT	60±11.7 (5)	NT	42 ± 18.6 (5)
10	50±6.4 (5)	45±7.0 (5)	75±10.0 (5)#	51±6.6 (5)	50±10.2 (5)	75±4.4 (5)*	68±7.1 (5)#	53±5.0 (5)

Results are average percentages (± SE) of total flies trapped using each lure; (n) =number of cages tested for each two-choice set up, containing one component and a solvent (hexane) control.

#- Wilcoxon Signed Rank Test, $P < 0.1–0.05$

*- Wilcoxon Signed Rank Test, $P < 0.05$

**- Wilcoxon Signed Rank Test, $P < 0.01$

NT - not tested

Attractive volatiles were collected from whole fruit aroma. Using a simple, though efficient, laboratory bioassay, we screened volatile preparations and demonstrated that the flies prefer volatiles from ripe melons that, in contrast to unripe fruit, are known to produce relatively large quantities of a wide variety of aromatic compounds (Beaulieu and Grimm 2001; Shalit et al. 2001).

The headspaces of ripe melons are characterized by volatile aldehydes, alcohols, and large quantities of acetate esters that are likely to be the key contributors to the unique aromas of melons (Shalit et al. 2001). GC-EAD of melon headspace revealed that the antennae of both sexes of *D. ciliatus* can detect at least 14 compounds in melon aroma, eight of which have been identified as acetates: benzyl acetate, hexanyl acetate, (*Z*)-3-hexenyl acetate, octanyl acetate, (*Z*)-3-octenyl acetate, (*Z*)-3-decenyl acetate, some (*E*)-3-decenyl acetate, and (*Z*)-3-dodecenyl acetate. The other identified compounds were isopentyl hexanoate, β-caryophyllene, (*E*)-β-farnesene, germacrene D, and cadinene (unknown isomer). Δ-Cadinene and β-caryophyllene were also described by Shalit et al. (2001) as components of the headspace of ripe Arava melon. Although the RI of the cadinene component in our samples was similar to that of Shalit et al. (2001), the MS spectrum matches better a structural isomer of γ-cadinene than δ-cadinene. Δ-Cadinene is known as one isomer; however, three γ-cadinene isomers are reported in the literature (Connolly and Hill 1991). It seems that the component in our sample is one of the

Table 3 Dose-dependent attraction of *D. ciliatus* males to 8 components of melon fruit volatiles

Amount (μg)	Benzyl acetate	Hexanyl acetate	(*Z*)-3-hexenyl acetate	Octanyl acetate	(*Z*)-3-octenyl acetate	(*E*)-3-decenyl acetate	(*Z*)-3-decenyl acetate	(*E*)-β-farnesene
0.125	61±4.9 (13)*	53±5.8 (5)	38±6.7 (5)#	49±3.5 (5)	51±5.4 (5)	42±4.7 (5)#	54±4.2 (10)	51±7.2 (5)
0.25	NT	NT	52±5.7 (5)	53±6.0 (5)	74±7.9 (5)*	61±6.9 (5)	57±6.8 (5)	56±5.8 (10)
0.5	48±3.3 (5)	68±7.5 (10)#	72±4.9 (5)*	64±7.3 (5)#	74±9.4 (5)#	57±4.6 (10)	62±4.4. (5)#	56±12.6 (5)
1	47±6.0 (5)	61±9.9 (5)	58±6.7 (10)	70±4.4 (10)**	58±2.8 (9)*	66±4.8 (10)*	57±7.1 (5)	61±4.6 (10)*
1.5	68±3.4 (10)**	NT	NT	60±4.8 (5)#	64±11.5 (5)	NT	54±4.6 (5)	NT
2	NT	56±5.4 (10)	NT	NT	NT	68±9.8 (5)	NT	48±3.9 (5)
10	76±6.1 (5)*	69±5.7 (5)*	49±6.0 (5)	47±6.7 (5)	50±8.5 (5)	75±7.2 (5)*	48±4.0 (5)	64±9.6 (5)

Results are average percentages (± SE) of total flies trapped using each lure; (n)= number of cages tested for each two-choice set up, containing one component and a solvent (hexane) control

#- Wilcoxon Signed Rank Test, $P < 0.1–0.05$

*- Wilcoxon Signed Rank Test, $P < 0.05$

**- Wilcoxon Signed Rank Test, $P < 0.01$

NT - not tested

isomers of γ-cadinene and not δ-cadinene. Unfortunately, the wide range of the RIs reported in the literature (NIST 5) and the lack of standards prevent determining the exact isomeric structure of our cadinene component.

Further EAG studies confirmed the specific sensing of most of the identified compounds. Both hexanyl acetate and (*E*)-β-farnesene previously have been described as elicitors of EAG responses in polyphagous flies, such as *C. capitata* and *Bactrocera dorsalis* (Light et al. 1988; Hull and Cribb 2001; Siderhurst and Jang 2006). Additionally, hexanyl acetate was identified as an elicitor of an EAG response in *Anastrepa ludens* (Malo et al. 2005).

The maximal responses of the antennae of both sexes of *D. ciliatus* were similar, occurring around 1.5 mV. However, the sexes differed in their sensitivity to the isolated compounds. While the responsiveness of males' antennae to hexanyl and benzyl acetate was greater than that of females, the females were more responsive to (*Z*)-3-hexenyl acetate. In behavioral bioassays, the dose response to the identified compounds was not clear. However, except for (*E*)-β-farnesene, the tested concentrations of all identified acetates were attractive to both sexes. *E*-β-Farnesene was deterrent to females but was slightly attractive to males. In fact, screening of several melon volatile collections revealed that (*E*)-β-farnesene is rather uncommon. Moreover, Nigg et al. (1994) reported that this compound is a deterrent to *A. ludens*, suggesting that it may play a role in the fruit flies' assessment of suitable fruits (i.e., as an indicator of host status). This finding, which may have some possible applications, merits further investigation.

Our results also revealed interesting differences in the behavioral responses of male and female *D. ciliatus* to the volatiles they were exposed to. As an example, females were consistently attracted to (*Z*) and (*E*)-3-decenyl acetate, while males were attracted to lower concentrations of (*Z*)-3-decenyl acetate, instead of the (*E*)-isomer. Also, both sexes were strongly attracted to (*Z*)-3-hexenyl, (*Z*)-3-octenyl, and octanyl acetates, but the attractiveness of the latter two was limited to doses below 10μg. The fact that males were more attracted to benzyl acetate than females could be explained

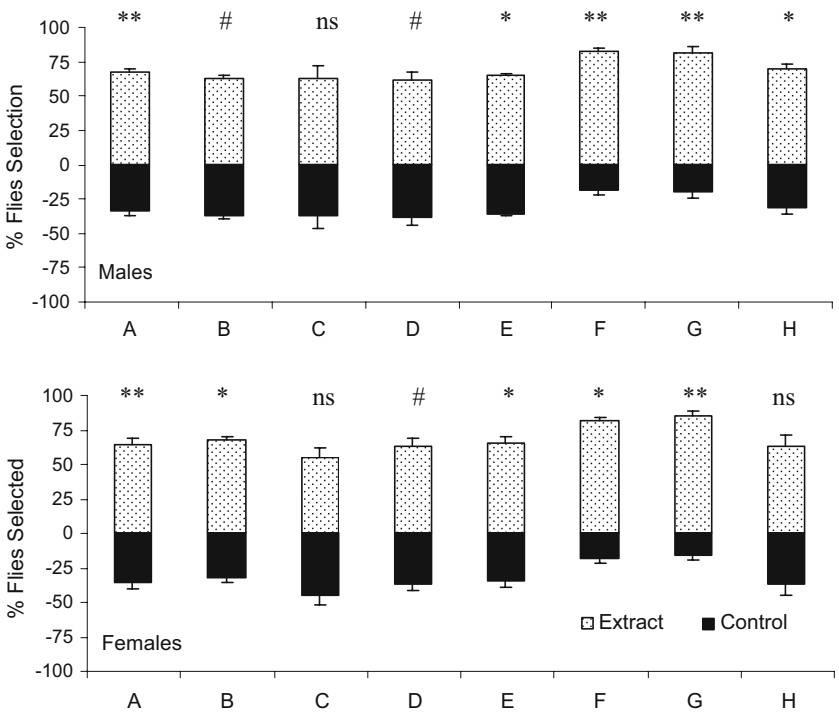

Fig. 4 Attraction of *D. ciliatus* to different mixtures of synthetic compounds (0.5 μg of each component per vial). Results are average percentages (±SE) of total flies trapped using each lure; *N*=number of cages tested for each two-choice set-up, consisting of a treatment mixture and a control (hexane); (a) males and (b) females. The response to the control is marked in *black*. The specific composition of each blend is indicated. **P*<0.05 Wilcoxon signed rank test, ** *P*<0.01 Wilcoxon signed rank test, # *P*<0.01 Wilcoxon signed rank test; *ns* not significant

Mix	A	B	C	D	E	F	G	H
Benzyl acetate								X
6 Ac				X			X	X
(*Z*)-3-6:Ac		X			X	X	X	X
8:Ac	X	X	X		X	X	X	X
(*Z*)-3-8:Ac	X	X	X		X	X	X	X
(*Z*)-3-10:Ac				X	X	X	X	X
(*E*)-3-10:Ac				X		X	X	X
(*E*)-β-farnesene			X					X
n	15	5	5	5	5	5	10	5

by males' higher antennal sensitivity to these compounds, as observed in EAG responses. However, there was no correlation between the antennal and behavioral responses in the cases of the other compounds.

At present, we do not have any explanation for the observed sexual differences in electrophysiological and behavioral responses to the examined volatile compounds. However, we suspect that the latter have evolved to fit sex-specific strategies and behaviors.

Despite the above-mentioned differences, the blend of identified compounds excluding benzyl acetate and (*E*)-β-farnesene (Fig. 4, blend G) proved attractive to both sexes. Excluding hexanyl acetate from the mixture (Fig. 4, blend F) did not reduce its attractiveness. The addition of benzyl acetate and (*E*)-β-farnesene to the blends decreased their attractiveness (Fig. 4, blend H), again strengthening the observation that (*E*)-β-farnesene seems to be a deterrent. However, we should keep in mind that, by using blends of compounds in equal proportions, we considerably raised the relative amount of (*E*)-β-farnesene, which is a minor component of melon emissions as observed in peak 9 of Fig. 2. Thus, under natural conditions, it is not expected to have negative effects. Since each of the six selected compounds were attractive and the difference in responses to the different blends was not high, it appears that the effect of blend components is not synergistic but may be additive. In any case, and based on the additive results observed for the blend, octanyl acetate and (*Z*)-3-octenyl acetate seem to be the two main identified attractants of *D. ciliatus*. These two compounds have not been reported previously as attractants for Tephritidae. On the other hand, hexanyl acetate was reported previously to be attractive to *B. dorsalis* (Siderhurst and Jang 2006), and benzyl acetate was attractive to males of the highly polyphagous *B. dorsalis*, *Bactrocera cucurbitae*, and *C. capitata* (Metcalf et al. 1986 and references therein).

Oligophagous flies, such as *D. ciliatus* that specialize on cucurbits, could be expected to respond to a narrow band of cues, presumably those specific to cucurbits. To address this issue, we need to evaluate volatile profiles of additional cucurbit hosts of *D. ciliatus*. However, our data indicate that a mixture of rather general volatiles is attractive to these flies. Thus, our results correspond well with the idea suggested by Bruce et al. (2005) for phytophagous insects, i.e., that the ratio-specific odor recognition, relying on a particular ratio of volatiles distributed generally among plants, is the more common mechanism of host recognition, rather than species-specific volatile–host recognition.

Our blends of six or seven acetates seem promising for field applications. However, we have to overcome the challenge of making a host-derived odor that is attractive under field conditions against a background of natural chemicals. In the case of *D. ciliatus*, the volatile profile of ripe melon may prove effective in fields of young, unripe melon. Moreover, if *D. ciliatus*, like the melon fly *B. cucurbitae*, exhibits daily movements among host plants and surrounding vegetation, attractants could be applied in perimeter traps, as suggested by Piñero et al. (2006). We currently are exploring this option. Our initial results indicate that blends may be attractive to the fly under field conditions (unpublished data). Other challenges include the need to understand the effect of fly physiological status (e.g., age and reproductive condition) on perception and attractiveness of the identified volatiles and incorporation of this information into a field application strategy. In particular, young and sexually immature females are expected to respond to cues differently from old and inseminated females. The former are looking for protein food sources for oogenesis (Kendra et al. 2005), whereas the latter may concentrate on searching for oviposition sites. Differential responses to volatiles may improve our understanding of their specific activity and may help to tailor appropriate blends, both for monitoring and control.

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Neonate Silkworm (*Bombyx mori*) Larvae Are Attracted to Mulberry (*Morus alba*) Leaves with Conspecific Feeding Damage

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Abstract The silkworm *Bombyx mori* is a molecular genetic model for the Lepidoptera. Its odorant receptor genes have been described, and preliminary studies suggest that several are expressed specifically in the larval caterpillar stage. This study was undertaken to identify olfactory behaviors specific to the larvae. A two-choice leaf disk bioassay with naive neonate larvae was used to evaluate the attractiveness of three types of mulberry leaf (*Morus alba*): newly flushed leaves from branch tips, mature leaves, and mature leaves with feeding damage caused by conspecific larvae. Mature leaves with feeding damage were the most attractive, newly flushed leaves were moderately favored, and undamaged mature leaves were the least attractive. Volatile odors collected from whole mulberry leaves by using solid-phase microextraction fibers were analyzed by gas chromatography-mass spectrometry. The volatile profile of newly flushed leaves and mature leaves damaged by conspecific larvae was more complex compared to undamaged mature leaves. By comparing the volatile makeup of each leaf type, a list of 22 candidate odors responsible for attracting the neonate larvae was generated; α-farnesene was particularly notable as a herbivore-induced volatile. These odors will be used in future in vitro studies to determine whether they activate larval-specific odorant receptors.

Keywords Silkworm · *Bombyx mori* · Mulberry · Leaf volatiles · Larval olfaction

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Introduction

Olfaction is one of the most important ways through which insects interact with their surroundings. Among its many functions, insects rely on chemoreception to locate both mates and food sources. For example, male Lepidoptera are able to locate females from long distances by sensing and orienting to female-produced sex pheromones (Schneider 1992). Female Lepidoptera use olfactory cues, including volatiles induced by feeding damage, to locate host plants when searching for oviposition sites (Renwick and Chew 1994). The silkworm *Bombyx mori* genome was sequenced in 2004 (Mita et al. 2004; Xia et al. 2004), and the silkworm is becoming a molecular genetic model species representing the Lepidoptera (Goldsmith et al. 2005). Approximately 50 different odorant receptor (Or) genes have been annotated from the silkworm genome (Wanner et al. 2007). Male-specific Ors activated by the sex pheromones bombykol and bombykal have been characterized (Sakurai et al. 2004; Krieger et al. 2005; Nakagawa et al. 2005) as well as female-biased Ors whose function remains uncertain (Wanner et al. 2007; Anderson et al. 2009). These studies represent initial efforts to link the molecular biology of insect olfaction with behavior and chemical ecology.

The females of many Lepidoptera species select the host plant for oviposition (Renwick and Chew 1994). However,

the hatching neonate larvae must contend with a variety of factors to locate and establish their feeding site, a behavior thought to be mediated primarily by olfaction (Zalucki et al. 2002). Many species of Lepidoptera disperse in the wind as early instars on silk threads, after which host plants and suitable feeding sites need to be located. Ablation experiments in the 1960s demonstrated that caterpillar antennae mediate olfactory attraction to odors located 10–30 cm distant (reviewed in Schoonhoven 1987). Due to its economic importance to sericulture, the olfactory behavior of silkworm larvae was studied extensively in the 1960s in Japan in relation to artificial diet and feeding behavior. Studies revealed that the terpenes found in mulberry leaves, such as citral, linalyl acetate, linalool, and terpinyl acetate, as well as terpene alcohols attract *B. mori* larvae (Hamamura and Naito 1961; Ishikawa et al. 1969). Citral and linalyl acetate were very attractive, while terpinyl acetate and hexanol were slightly less attractive.

The volatile profile of a plant often changes as a result of herbivore feeding damage (Dicke et al. 1988; Turlings et al. 1995; Pare and Tumlinson 1999; Kessler and Baldwin 2001). Herbivore-induced volatiles differ from those produced by mechanical damage (Dicke et al. 1988; Pare and Tumlinson 1999). The volatiles that are produced in response to herbivore feeding are often attractive to predators and parasites of the herbivores that induced the damage (Pare and Tumlinson 1999; Hoballah and Turlings 2005). The signaling function of the volatiles may have evolved as a secondary function of compounds that function to deter herbivore feeding behavior and prevent infection of damaged tissue (Turlings et al. 1995). By attracting predators, plants may be protected against further damage.

Herbivores themselves can use herbivore-induced volatiles as chemical cues, but this subject has been less well studied. Caterpillar feeding during the day can induce plant volatiles at night that deter oviposition by conspecific moths (De Moraes et al. 2001). Some lepidopteran larvae are attracted to food sources with conspecific feeding damage. Apple codling moth (*Cydia pomonella*) larvae are preferentially attracted to volatiles from apples that have been infested by other codling moth larvae compared to undamaged or mechanically damaged apples (Landolt et al. 2000). Similarly, fall armyworm (*Spodoptera frugiperda*) neonate larvae are more attracted to cowpea leaves damaged by conspecifics than to undamaged cowpea leaves (Carroll et al. 2008).

The order Lepidoptera includes many significant pest species of crop plants worldwide, and it is the larval caterpillar stage that causes feeding damage. Molecular genetic approaches to understanding host-seeking and selection behavior by larvae may help provide a foundation for the development of new pest management techniques. For example, plants have been genetically modified to produce volatiles that attract aphid parasitoids (Beale et al. 2006). Preliminary research has identified several silkworm Ors that appear to be expressed specifically in the larval antennae and not in adult antennae.

This study was conducted to identify larval-specific silkworm behaviors and the odors that mediate these behaviors to provide a basis to further study the molecular function of larval-specific Ors. The volatile profiles of three different categories of mulberry leaves (newly flushed leaves, mature leaves, and mature leaves with conspecific feeding damage) and their attractiveness to neonate silkworm larvae were evaluated.

Methods and Materials

Leaf Disk Assays White mulberry (*Morus alba*) leaves were collected daily (Champaign-Urbana, Illinois area) in the early morning typically between 7:00 and 9:00 A.M. each day that tests were conducted. Following collection, leaf disks were cut with an 18-mm cork borer. Three categories of leaf disk were prepared: mature (dark green) leaves, newly flushed (light green) leaves collected from branch tips, and mature leaves with feeding damage. Mature leaf disks with and without feeding damage were prepared by placing cut disks in a 25-mm Petri dish (lined with moistened filter paper to prevent desiccation) for 24 h. One set of Petri dishes included five neonate larvae that were allowed to feed for the 24-h-period. Prior to testing, the larvae and frass were removed. In some choice tests, the filter paper underneath the mulberry leaf disks also were used after it was cleaned of frass and larvae and folded into a small (approximately 1 cm) bundle. Filter paper underneath mature leaf disks without larval feeding was prepared by using the same procedure.

All choice tests were conducted with the same procedure, changing only the odor sources. Each replicate tested the odor preferences of naive neonate *B. mori* larvae that had hatched within 24 to 48 h. A batch of 10,000 silkworm eggs was purchased from Mulberry Farms (Fallbrook, CA, USA) and stored at 4°C. Larvae hatched from eggs as needed in plastic Petri dishes without food. Glass Petri dishes (80 mm diameter) were washed with dilute Liqui-Nox detergent (Fisher Scientific), rinsed with acetone, and baked at 240°C for at least 2 h before being used in choice tests. Two odor sources were placed on opposite sides of the glass Petri dish, and five neonate larvae were placed in the center of the dish with the lid in place. Larvae were allowed to crawl for 15 min after which their positions were noted (at odor source 1, at odor source 2, or in-between). If all five larvae reached an odor source before 15 min had expired, the test was stopped, and the position of the larvae

was noted. Two assays (five larvae per assay) were performed each day on five different days, for a total of ten replicates of five larvae in each replicate.

Six comparisons were conducted using leaf disks from: (1) mature leaves vs. mature leaves, (2) mature leaves vs. newly flushed leaves, (3) mature leaves vs. mature leaves with feeding damage, (4) mature leaves with feeding damage vs. newly flushed leaves, (5) filter paper underneath mature leaves vs. filter paper underneath mature leaves with feeding damage, and (6) mature leaves with feeding damage vs. the filter paper underneath mature leaf disks with feeding damage. The null hypothesis that equal proportions of larvae chose each type of odor source was tested with the chi-square statistic. Choice tests with single larvae also were conducted to rule out the possibility that larvae were interacting with each other when selecting the odor source.

Solid-Phase Microextraction Fiber Assay To collect the volatiles released from each type of mulberry leaf, a solid-phase microextraction fiber was used (SPME, Supelco Bellefonte, PA, USA; the fiber was coated with polydimethylsiloxane/divinylbenzene). Whole leaves with petioles attached were placed in a 125-ml Erlenmeyer flask and covered with aluminum foil. Flasks were thoroughly washed with dilute Liqui-Nox detergent, rinsed with acetone and baked for at least 2 h at 240°C between each use. The SPME fiber was inserted through the foil and exposed 2–4 cm from the surface of the sample material for 30 min. A 50-W full-spectrum plant light was directed at the flask from 30 in. away. The temperature of the flask was monitored and never exceeded 30°C.

Volatiles were collected from each sample material (12 g of whole leaves with petioles) on three different days. Newly flushed mulberry leaves are smaller and weighed less per leaf, and 10 g of leaves were analyzed for each replicate. Mature leaves with feeding damage were prepared by placing 12 g of whole mature leaves with petioles in a 125-ml flask with approximately 100–200 neonate larvae that were allowed to feed overnight, after which the larvae, frass, and silk were removed prior to volatile collection. Immediately after the 30-min exposure to the test material, the SPME fibers were analyzed with a gas chromatograph-mass spectrophotometer, GC-MS (HP Agilent 6890 Gas Chromatograph and HP 5973 Mass Spectrometer, Palo Alto, CA, USA), fitted with an HP-5MS column. The fiber was heated to 40°C for 1 min, increased 10°C/min to 250°C, and then held at 250°C for 5 min. SPME fibers were blanked (baked at 250°C for 5 min) immediately prior to collecting volatiles.

Volatile peaks that were identified with at least 80% confidence by Enhanced MSD Chemstation (version D.02.00.275, Agilent Technologies) software and the NIST Mass Spectral Database were considered candidates. The identity of the candidate volatiles were confirmed with standards purchased from Sigma-Aldrich (St. Louis, MO, USA) with purities typically greater than 98%. Small amounts of the pure standards were spotted onto filter paper in a flask, exposed to a SPME fiber for 5 min, and analyzed on the GC-MS using the same program. The identity of the leaf volatiles was confirmed if the retention times and mass spectra of the standard chemical and the peak in leaf volatile samples matched.

Results

Leaf Disk Assays To evaluate the two-choice leaf disk assay, two similar samples were compared (leaf disks cut from mature mulberry leaves). The naive neonate silkworm larvae did not exhibit any bias; equal proportions selected each of the two similar samples (chi-square=0.03, P=0.87). The same results were obtained when larvae were tested individually, supporting the subsequent use of larvae in groups of five (unpublished results). When presented with similar choices, a greater proportion of larvae failed to select either sample within the 15-min assay (Fig. 1).

Larvae demonstrated a clear preference for leaf disks cut from newly flushed leaves (74%) compared to disks from mature leaves (20%; Fig. 1, chi-square=15.5, P<0.001). The same preference for newly flushed leaves was demonstrated in preliminary experiments that used individual larvae (unpublished results). Mature leaf disks with conspecific feeding damage also were clearly preferred (80%) compared to mature leaf disks with no damage (12%; chi-square=25.1, P<0.001). Larvae preferred mature leaf disks with conspecific feeding damage (60%) compared to newly flushed leaf disks (32%), but the difference was less pronounced (Fig. 1, chi-square=4.26, P=0.04). In all three of these choice experiments, more than 92% of the larvae chose one of the treatments within 15 min.

Surprisingly, the moist filter paper used to keep leaf disks with feeding damage from desiccating was highly attractive in preliminary choice assays. We assayed the attractiveness of filter paper relative to mature leaf disks with feeding damage, the most attractive leaf type. Filter paper underneath leaf disks with feeding damage was significantly preferred (76%) compared to the filter paper (12%) underneath mature leaves with no feeding damage (Fig. 1, chi-square=23.3, P<0.001). Mature leaf disks with feeding damage appeared to be only slightly more attractive (50%) when compared to filter paper (38%) that they were placed on top off, and the difference was not statistically significant (chi-square=0.82, P=0.37). The odor(s) that

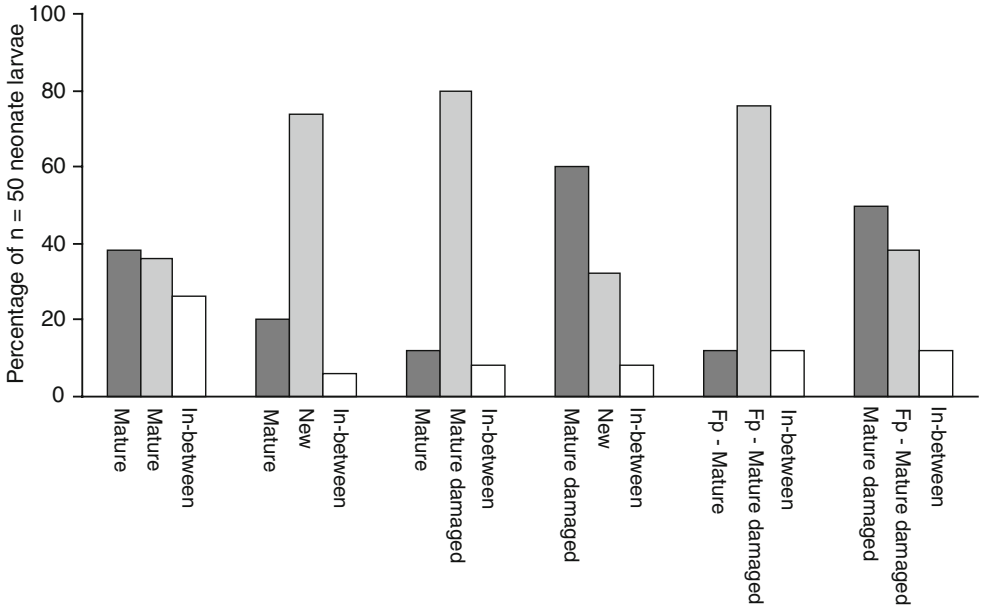

Fig. 1 Attraction of neonate silkworm larvae to different mulberry leaf types. Percentage *of N*=50 neonate larvae attracted to two different leaf disk types in six different choice experiments: (1) mature leaves vs. mature leaves (chi-square 0.03, P=0.87), (2) mature leaves vs. newly flushed leaves (chi-square 15.5, P<0.001), (3) mature leaves vs. mature leaves with feeding damage (chi-square 25.1, P< 0.001), (4) mature leaves with feeding damage vs. newly flushed leaves (chi-square 4.26, P=0.04), (5) filter paper exposed to mature leaves (*Fp-mature*) vs. filter paper exposed to mature leaves with feeding damage (*Fp-mature damage*; chi-square 23.3, P<0.001), and (6) mature leaves with feeding damage vs. the filter paper exposed to mature leaf disks with feeding damage (chi-square 0.82, P=0.37). Larvae that did not exhibit a preference after 15 min were termed in-between

absorbed into the filter paper were highly attractive even when compared to the most attractive leaf type.

SPME Fiber Assay SPME analysis of whole mulberry leaves with petioles attached (conducted in triplicate) yielded different volatile profiles for each category (Fig. 2, Table 1). Figure 2 illustrates the volatile profile of a typical replicate for each leaf type, and Table 1 lists the identities (confirmed using standard reference chemicals) and their proportional abundance. The mature mulberry leaves yielded a relatively simple volatile profile with two predominant peaks (#2 and #5) representing (*Z*)-3-hexen-1-ol and an unidentified compound. A third less abundant peak, #16, was identified as (*Z*)-3-hexenyl butyrate (Fig. 2, Table 1). These three volatiles were detected in all three leaf types; (*Z*)-3-hexen-1-ol and the unidentified peak were predominant, while (*Z*)-3-hexenyl butyrate was less abundant, particularly in newly flushed leaves.

Newly flushed leaves also produced a greater number of minor peaks yielding a more complex volatile profile (peaks #8–11, 13–15, and 20–22). However, with the exception of #13 ((*Z*)-β-ocimene), the abundance of these minor peaks (identified as a variety of terpenes including α-terpinene, *o*-cymene, D-limonene, eucalyptol, γ-terpinene, linalool, caryophyllene, and β-cubebene) was measurable in only one of the three replicates (Table 1). For example, while minor peaks #8 to 11 were identified in Fig. 2b, their abundance was below the software's detection threshold in all but one replicate. The proportional abundance of these minor peaks ranged from 1.1% to 4.4% of the total volatiles (percent of the total peak area, Table 1). The variability between replicates may be related to variability in the developmental stage of newly flushed leaves collected in the field.

Mature mulberry leaves with conspecific feeding damage produced more complex volatile profiles that were consistent between replicates (Table 1). In addition to the three common volatiles (peaks #2, 5, and 16), several short-chain alcohols, aldehydes, and acetates were produced by leaves with feeding damage, including (*E*)-2-hexenal, (*E*)-2-hexen-1-ol, 1-hexenol, hexyl acetate, and (*E*)-2-hexenyl acetate (peaks #1, 3, 4, 6, and 7). Other volatiles that were detected from leaves with feeding damage included eucalyptol, (*E*)-β-ocimene, (*Z*)-β-ocimene, linalool, (*Z*)-3-hexenyl butyrate, hexyl butyrate, methyl salicylate, (*Z*)-3-hexenyl 2-methylbutanoate, caryophyllene, β-cubebene, and α-farnesene (peaks #11–13, 15–22, Fig. 2 and Table 1). The abundance of eucalyptol, (*E*)-β-ocimene, methyl salicylate, and caryophyllene was measurable in one replicate only. Several of these volatiles may be associated with leaves that have feeding damage, including (*E*)-2-hexenal, (*E*)-2-hexen-1-ol, 1-hexenol, hexyl acetate, (*E*)-2-hexenyl acetate, (*E*)-β-ocimene, hexyl butyrate, methyl salicylate, (*Z*)-3-hexenyl 2-methylbutanoate, and α-

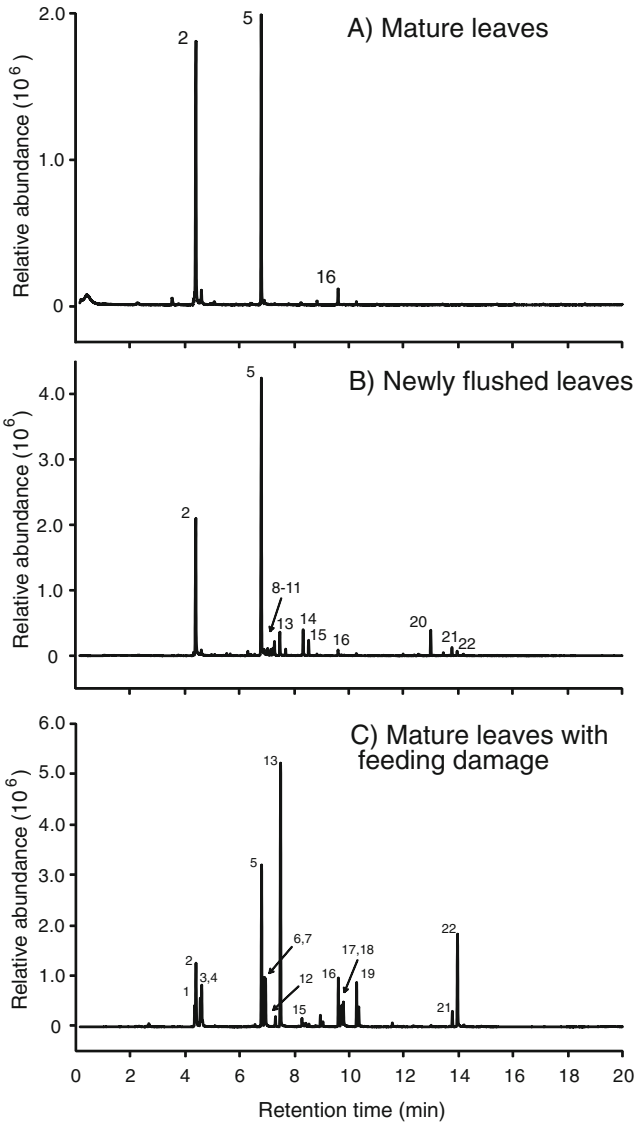

Fig. 2 Volatile profile of three mulberry leaf types. **a** Mature leaves, **b** newly flushed leaves, and **c** mature leaves with feeding damage from neonate silkworms. Each of the selected total ion current chromatograms are representative of three replicated experiments. The numbered peaks correspond to compounds identified in Table 1. Retention time (min) is labeled on the *x-axis*, relative abundance ($\times 10^6$) on the *y-axis*

farnesene (Table 1). Of particular note, α-farnesene was produced consistently in all three replicates of feeding-damaged leaves (proportional abundance ranged from 6% to12%), indicating that it may function as a herbivore-induced volatile, much as it is known to act in the apple coddling moth/apple tree system (Landolt et al. 2000). A small peak corresponding to α-farnesene was detected from newly flushed leaves, but its abundance was below threshold levels. Also notable, methyl salicylate was detected as a volatile specific to herbivore-damaged mulberry leaves although in one replicate only. Methyl salicylate is an important mobile signal for plant systemic acquired resistance (Park et al. 2007), and its ability to attract beneficial insects has been tested in field trials (James and Price 2004).

Discussion

Using a two-choice leaf disk assay, naive neonate silkworm larvae were found to be strongly attracted to mature mulberry leaves with conspecific feeding damage and also to newly flushed leaves, compared to undamaged mature leaves. The neonate larvae of Lepidoptera species commonly feed on newly flushed foliage, presumably because it is easier to digest, providing a plausible biological rationale for the attraction of neonate silkworms to newly flushed mulberry leaf disks. The attraction of neonate silkworms to mulberry leaves with conspecific feeding damage was strong and more difficult to explain. The behavior was robust enough that larvae placed on a lab bench all crawled towards leaves with feeding damage rather than undamaged leaves. Neonate apple codling moth (*C. pomonella*) larvae and neonate fall armyworm (*S. frugiperda*) larvae also have been found to be attracted to host plants damaged by conspecific feeding (Landolt et al. 2000; Carroll et al. 2008). The emission of specific plant volatiles in response to insect feeding damage and their role in attracting and recruiting natural enemies of the herbivore has been well documented (Dicke et al. 1988; Turlings et al. 1995; Pare and Tumlinson 1999; Kessler and Baldwin 2001; Hoballah and Turlings 2005). However, the response of the herbivores themselves to the volatiles induced by their feeding has not been as well studied (De Moraes et al. 2001). Locating and establishing suitable feeding sites is critical to the survival of neonate larvae (Zalucki et al. 2002). Larval aggregation in the Lepidoptera is not uncommon, and it is believed to confer advantages such as increasing foraging efficiency and aiding in defense (Zalucki et al. 2002). Neonate larvae have limited searching capacity, and damaged tissues that emit more volatiles might be easier to detect (Zalucki et al. 2002). Herbivore-induced volatiles may also provide information about host plant quality (Carroll et al. 2006). Conspecific feeding may be a marker of habitat fitness on the basis that other individuals have found a particular location to be suitable for feeding (Stamps and Krishnan 2005). Furthermore, when the concentration of conspecifics in an area increases, their ability to overcome plant defenses may increase (Prokopy and Roitberg 2001). Other behavioral aspects of caterpillar aggregation have been discussed recently in Carroll et al. (2006) and Carroll et al. (2008).

Compared to mature undamaged leaves, the volatile profile of newly flushed mulberry leaves and mature leaves

Table 1 GC-MS analysis of mulberry leaf volatiles collected by using SPME fibers

Peak #	Compound	Retention Time (min)	Proportion (%) of total peak area[a] (mean ± S.D.)		
			Mature	New	Eaten
1	(*E*)-2-Hexenal	4.35			3.9±3.6
2	(*Z*)-3-Hexen-1-ol	4.40	28.1±23.2	36.3±12.9	13.2±8.8
3	(*E*)-2-Hexen-1-ol	4.56			5.5±4.0
4	1-Hexenol	4.61			4.3±4.1
5	Unknown[b]	6.79	67.4±21.1	49.4±5.4	18.0±8.0
6	Hexyl acetate	6.90			2.3±1.4
7	(*E*)-2-Hexenyl acetate	6.94			3.5±0.9
8	α-Terpinene	7.02		(4.2)	
9	o-Cymene	7.15		(4.4)	
10	D-Limonene	7.23		(1.1)	
11	Eucalyptol[b]	7.23		(2.4)	(1.4)
12	(*E*)-β-Ocimene[b]	7.30			(0.6)
13	(*Z*)-β-Ocimene[b]	7.48		4.4±0.9	17.2±1.1
14	γ-Terpinene	7.69		(4.3)	
15	Linalool	8.32		(3.9)	0.7±0.4
16	(*Z*)-3-Hexenyl butyrate	9.60	4.4±3.0		2.6±0.8
17	Hexyl butyrate	9.69			1.1±0.4
18	Methyl salicylate	9.79			(1.6)
19	(*Z*)-3-Hexenyl 2-methylbutanoate[b]	10.27			14.7±22.6
20	β-Caryophyllene	13.00		(4.2)	(1.0)
21	β-Cubebene[b]	13.78		(1.5)	0.8±0.04
22	α-Farnesene	13.97			9.1±3.2

Volatiles were collected and identified from three types of whole Mulberry leaf: (a) mature dark green leaves, (b) newly flushed light green leaves from branch tips, and (c) mature leaves with feeding damage from neonate silkworms. The abundance of each volatile is reported as a proportion of the total peak area. Numbered volatiles correspond to the peak numbers on the total ion current chromatograms in Fig. 2. The identity of 16 volatiles was verified by using standard reference chemicals with purities typically greater than 98%

[a] Bracketed numbers indicate that the volatile was detected in only one of three replicates

[b] Six volatiles for which standard chemicals could not be obtained

damaged by conspecific feeding was more complex. Of the 22 volatiles identified, two green leaf volatiles were common to most samples, (*Z*)-3-hexen-1-ol, and (*Z*)-3-hexenyl butyrate. Several other green leaf volatiles (6-carbon alcohols, acetates, and aldehydes), including (*E*)-2-hexenal, (*E*)-2-hexen-1-ol, hexyl acetate, (*E*)-2-hexanyl acetate, and hexyl butyrate were detected only from whole mulberry leaves that had sustained feeding damage from neonate silkworms for a period of approximately 18 h. Green leaf volatiles typically are released quickly after mechanical damage caused by biotic or abiotic factors. Green leaf volatiles associated with fresh mechanical damage subside quickly, and herbivore-induced volatiles such as terpenoids produced several hours later are indicative of older damage (Hoballah and Turlings 2005). The mulberry leaves used in this study had sustained feeding damage continuously up until the time that volatiles were sampled with SPME fibers, yielding a mixed fresh and older damage volatile profile.

In addition to the green leaf volatiles, several terpenoids were identified from leaves with feeding damage (Table 1). These included eucalyptol, (*E*)- and (*Z*)-β-ocimene, linalool, caryophyllene, β-cubebene, caryophyllene, and α-farnesene. Several terpenoids were identified from newly flushed leaves but at low abundance and inconsistently between replicates. Among these candidate odors, α-farnesene may be one of the more interesting volatiles since it was produced consistently by mulberry leaves with feeding damage. Neonate apple coddling moth larvae are attracted to α-farnesene, which is produced in response to feeding damage by conspecific larvae (Landolt et al. 2000). In our experiments, moist filter paper placed underneath the leaf disks with feeding damage also was highly attractive. A preliminary GC-MS analysis of the filter paper volatiles (Supple-

mentary Fig. 1) yielded α-farnesene as the predominant peak.

Also interesting was the fact that methyl salicylate, involved in resistance signaling in plants (Park et al. 2007), was detected only from mulberry leaves with feeding damage (however, in one replicate only). Recent research indicates that methyl salicylate also may influence the behavior of beneficial insects (Williams et al. 2008). However, when a variety of pure volatiles was tested in the two-choice assay (unpublished results), larval responses were weak and difficult to interpret. Linalool, abundantly induced by fall armyworm feeding on maize seedlings, was attractive to sixth instars on its own or as a supplement to whole plant odors (Carroll et al. 2006). To simulate a natural blend, we used mature leaf disks spiked with a range of concentrations of the candidate volatiles. However, we could not demonstrate a clear attraction to any one volatile. A blend of volatiles or more natural release rates may be required to replicate the attraction.

The attraction of neonate silkworms to mulberry leaves damaged by feeding occurred in the absence of prior feeding or olfactory experience with the host plant, suggesting that the behavior is innate. The attraction of neonate fall armyworms to cowpea seedlings also occurred in the absence of prior experience (Carroll et al. 2008). Neonate larvae have a limited time in which to successfully locate and establish a feeding site before starving. Pest management strategies that alter the chemosensory profile of the host plant might have potential for exploiting this vulnerable stage. Chemical cues that stimulate neonate dispersal from the host plant may increase mortality rates (e.g., (Varela and Bernays 1987; Carroll et al. 2006).

The fact that silkworms are attracted to herbivore-induced volatiles provides an opportunity to study the molecular mechanisms that underlie this behavior. The silkworm genome has been sequenced, and genetic tools continue to be developed, making it one model species that represents the Lepidoptera. Some *Drosophila* Ors are expressed specifically in the larval stage (Kreher et al. 2005) suggesting they detect odors that mediate larval-specific behaviors. The candidate odors identified in this study will be used as ligands to characterize the function of larval-specific silkworm Ors. Many of the molecular mechanisms of host seeking and selection behavior in the Lepidoptara are likely to be conserved, and research that used model species, such as the silkworm, can be applied to economically significant pest species.

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Gustatory Responsiveness to Six Bitter Tastants in Three Species of Nonhuman Primates

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Abstract Gustatory responsiveness of six adult squirrel monkeys, four spider monkeys, and five pigtail macaques to six bitter tastants was assessed in two-bottle preference tests of brief duration (2 min). Animals were given the choice between a 30-mM sucrose solution and defined concentrations of a bitter tastant dissolved in a 30-mM sucrose solution. With this procedure, *Saimiri sciureus*, *Ateles geoffroyi*, and *Macaca nemestrina* were found to significantly discriminate concentrations as low as 0.2, 0.05, and 0.1 mM quinine hydrochloride; 1, 1, and 0.05 mM caffeine; 20, 5, and 1 mM naringin; 5, 2, and 1 mM salicin; 0.01, 0.001, and 0.02 mM sucrose octaacetate; and 0.05, 0.01, and 0.5 mM denatonium benzoate, from the alternative stimulus. With the exception of naringin in the pigtail macaques, all three species rejected all suprathreshold concentrations of all bitter tastants tested. The spider monkeys and the pigtail macaques displayed the lowest taste avoidance thresholds with three of the six tastants each; in contrast, the squirrel monkeys displayed the highest taste avoidance thresholds with four of the six tastants. The across-tastant patterns of taste avoidance thresholds were identical in spider monkeys and squirrel monkeys; both species displayed the following order of sensitivity: sucrose octaacetate > denatonium benzoate > quinine hydrochloride > caffeine > salicin > naringin. All three primate species were more sensitive to the two artificial tastants (sucrose octaacetate and denatonium benzoate) compared to the four naturally occurring tastants. However, the concentrations detected by all three primate species with the four naturally occurring tastants are well below those reported in plants or arthropods consumed by these species suggesting that they may use bitterness as a criterion for food selection.

Keywords Taste preference thresholds · Bitter taste · *Saimiri* · *Ateles* · *Macaca*

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Introduction

The sense of taste has been investigated behaviorally (e.g., Glaser 1986; Pritchard et al. 1994; Hladik et al. 2002) and electrophysiologically (e.g., Hellekant and Ninomiya 1994; Scott et al. 1998, 1999) in a number of primate species. However, most studies have concentrated on detectability of the five basic taste qualities, mainly by using the alkaloid quinine hydrochloride as the only prototypic bitter stimulus. Only sparse information is available on the behavioral responsiveness of nonhuman primates to naturally occurring bitter tastants other than this alkaloid. This is all the more surprising given the wide variety of chemical classes that comprise naturally occurring bitter-tasting substances (DuBois 2007) and given that the vast majority of food-related toxins taste bitter to humans (Rouseff 1990). Further, both plants and arthropods frequently use bitter-tasting and often highly toxic compounds as deterrents against potential predators (Belitz and Wiester 1985; Eisner et al. 2005). Therefore, it should be adaptive for primates to detect bitter tastants at concentrations low enough to prevent ingestion of too high amounts of such potentially toxic compounds.

Given the presumed importance of bitter tastants for food selection in primates, it was the aim of the present

study to assess the gustatory responsiveness of three species of nonhuman primates to six bitter tastants belonging to different chemical classes. Four of these tastants, quinine hydrochloride, caffeine, naringin, and salicin, occur naturally in both plants and arthropods, whereas the other two, sucrose octaacetate and denatonium benzoate, have not been found so far in nature (Belitz and Wiester 1985).

As all primate species tested so far reject substances that taste bitter to humans, a two-bottle preference test of brief duration (2 min) was employed. This method makes it possible to measure directly preferences for or aversions to tastants and largely rules out the possibility of postingestive factors on the animals' ingestive behavior. Further, as all three primates used in the present study cooperated in tests of liquid consumption only as long as at least one of the alternatives was a sapid solution, we decided to use a 30-mM sucrose solution rather than water, both as the solvent for the tastant and as the alternative stimulus. This concentration of sucrose is only a factor of 3 above the taste preference threshold of the squirrel monkeys and the pigtail macaques, respectively, and a factor of 10 above that of the spider monkeys and thus represents a weak sweet stimulus that is unlikely to mask the bitter tastant.

The three primate species employed here, squirrel monkeys (*Saimiri sciureus*), spider monkeys (*Ateles geoffroyi*), and pigtail macaques (*Macaca nemestrina*), are known to differ—at least to some degree—in their dietary habits (Caldecott 1986; Lima and Ferrari 2003; Gonzalez-Zamora et al. 2009), thus allowing us to address the question whether possible differences in bitter taste responsiveness may reflect an evolutionary adaptation to dietary specialization. The ability to include two New World and one Old World primate species (the macaques) allowed us to address additionally the question whether the degree of phylogenetic relatedness rather than dietary specialization may affect the bitter taste responsiveness of nonhuman primates.

Methods and Materials

Animals Testing was carried out with six male adult squirrel monkeys (*S. sciureus*), two male and three female adult pigtail macaques (*M. nemestrina*), and four female adult spider monkeys (*A. geoffroyi*). Animals were housed as social groups in enclosures with adjacent single cages that could be closed by sliding doors to allow temporary separation of animals for individual testing (for details of maintenance, see Laska 1996, 2000; Laska et al. 1996). All animals were born in captivity and had no experience with natural vegetation. They were fed commercial monkey chow, fresh fruit, and vegetables ad libitum but were deprived of water overnight before testing on the following morning. The amount of food offered daily to the animals was such that leftovers still were present on the floor the next morning. Thus, it was unlikely that ravenous appetite affected the animals' ingestive behavior during the tests.

Taste Stimuli A set of six substances tasting bitter to humans was used: quinine hydrochloride (quinine is a naturally occurring alkaloid in the bark of cinchona trees; as it is a basic amine, it is presented as a salt, in this case as a hydrochloride, Chemical Abstracts Service # 130-89-2), caffeine (a naturally occurring xanthine alkaloid, e.g., in coffee plants, CAS# 58-08-2), naringin (a naturally occurring flavonoid glycoside, e.g., in grapefruit, CAS# 10236-47-2), salicin (a naturally occurring phenol glycoside in many Salicaceae, CAS# 138-52-3), sucrose octaacetate (an artificial acetylated sucrose derivative, CAS# 126-14-7), and denatonium benzoate (an artificial compound with the International Union of Pure and Applied Chemistry name phenylmethyl-[2-[(2,6-dimethylphenyl)amino]-2-oxoethyl]-diethylammonium benzoate, CAS# 3734-33-6). The rationale for using these substances was that they represent different chemical classes and are naturally occurring as well as artificial bitter tastants. All substances were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were of the highest available purity.

Procedure Taste responsiveness to the six bitter tastants mentioned above was assessed by using a two-bottle preference test of short duration (Richter and Campbell 1940). Twice each day, approximately 2 and 1 h before feeding, the animals were separated and allowed 2 min to drink from a pair of simultaneously presented graduated cylinders with metal drinking spouts.

Animals were given a choice between a 30-mM sucrose solution and defined concentrations of the bitter tastants dissolved in a 30-mM sucrose solution. With quinine hydrochloride, caffeine, naringin, and salicin, testing started at a concentration of 10 mM and proceeded in the following steps (10, 5, 2, 1, 0.5, 0.2, 0.1 mM, etc.) until the animals failed to show a significant preference or aversion. With sucrose octaacetate and denatonium benzoate, testing started at a concentration of 1 mM and proceeded in the following steps (1, 0.5, 0.2, 0.1, 0.05 mM, etc.).

To maintain the animals' motivation and willingness to cooperate, testing did not follow a strict order of increasing or decreasing bitter tastant concentrations but followed a pseudorandomized scheme in which trials with taste mixtures containing high and thus presumably aversive concentrations of a bitter tastant were alternated with mixtures containing low and thus presumably less aversive concentrations of the same bitter tastant.

Each pair of stimuli was presented a total of ten times, and the position of the stimuli was pseudorandomized in

order to counterbalance possible position preferences. All animals had served in previous studies that use the same method (Laska and Hernandez Salazar 2004, Laska et al. 2008). They were trained to enter the single cages voluntarily and were completely accustomed to the procedure.

The experiments reported here comply with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication no. 86-23, revised 1985) and also with current German and Mexican laws.

Data Analysis For each animal, the amount of liquid consumed from each bottle was recorded, summed for the ten test trials with a given stimulus combination, converted to percentages (relative to the total amount of liquid consumed from both bottles), and 66.7% (that is, two thirds of the total amount of liquid consumed) was taken as criterion of preference. This rather conservative criterion was chosen for reasons of comparability of data, as the same criterion had been used in previous studies that used the same method with the same primate species (Laska 1994, 1996, 1997, 1999, 2000; Laska et al. 1996, 1998, 1999, 2000a, b, 2001, 2008; Laska and Hernandez Salazar 2004), and in order to avoid misinterpretation of data due to a too liberal criterion.

Additionally, two-tailed binomial tests (Siegel and Castellan 1988) were performed, and an animal was only regarded as significantly preferring one of the two alternative stimuli if it reached the criterion of 66.7% and consumed more from the bottle containing the preferred stimulus in at least eight out of ten trials (binomial test, $P< 0.05$). Accordingly, at the group level, a given species was regarded only as significantly preferring one of the two alternative stimuli if all animals tested reached the criterion of 66.7%, and all animals consumed more from the bottle containing the preferred stimulus in at least eight out of ten trials.

Preliminary tests showed that the animals generally rejected solutions containing relatively high concentrations of the bitter tastants. Following convention, the results nevertheless are expressed as percentage preference for the tastant and not for the solvent. Accordingly, 33.3% (that is, one third of the total amount of liquid consumed) was taken as criterion of aversion.

Preliminary analysis of the data indicated that there were no reliable differences in choice behavior and liquid consumption between males and females of a species and between the first and the second presentation of the day. Intraindividual variability of the amount of liquid consumed across the ten test trials with a given stimulus combination was low and averaged less than 20%. Thus, a theoretically possible bias in the overall preference score due to excessive drinking in aberrant trials did not occur. Therefore, data obtained in ten test trials from an individual animal of a given species with a given stimulus combination were combined and are reported as group means and standard deviations.

Results

Figures 1, 2, and 3 show the mean taste responses of squirrel monkeys, spider monkeys, and pigtail macaques, respectively, in the two-bottle preference tests with a 30-mM sucrose solution used both as solvent for the bitter tastants and as the alternative stimulus.

Squirrel monkeys significantly discriminated concentrations as low as 0.2 mM quinine hydrochloride, 1.0 mM caffeine, 20 mM naringin, 5 mM salicin, 0.01 mM sucrose octaacetate, and 0.05 mM denatonium benzoate from the alternative stimulus (group level). In most cases, interindividual variability of scores was low, as can be inferred from the SDs in Fig. 1. Thus, at the individual level, the avoidance threshold values for a given tastant maximally differed by a factor of 5 (quinine hydrochloride, naringin, and denatonium benzoate), and usually less, between subjects.

At the group level, the spider monkeys significantly discriminated concentrations as low as 0.05 mM quinine hydrochloride, 1.0 mM caffeine, 5 mM naringin, 2 mM salicin, 0.001 mM sucrose octaacetate, and 0.01 mM denatonium benzoate from the alternative stimulus. As for the squirrel monkeys, interindividual variability of scores was usually low (compare SDs in Fig. 2) and avoidance threshold values for a given tastant maximally differed by a factor of 5 (quinine hydrochloride), and in one case (caffeine) not at all, between subjects.

Pigtail macaques showed significant discrimination of concentrations as low as 0.1 mM quinine hydrochloride, 0.05 mM caffeine, 1 mM naringin, 1 mM salicin, 0.02 mM sucrose octaacetate, and 0.5 mM denatonium benzoate from the alternative stimulus (group level). Again, interindividual variability of scores was low in most cases (compare SDs in Fig. 3). Thus, at the individual level, the avoidance threshold values for a given tastant maximally differed by a factor of 5 (caffeine), and in two cases (quinine hydrochloride and denatonium benzoate) not at all, between subjects. Interestingly, the pigtail macaques preferred rather than rejected naringin at concentrations between 10 and 1 mM.

With all six bitter tastants and all three species, we observed that, in each trial, each animal sampled both alternatives at least once and, with the exception of the highest bitter tastant concentrations, even tended to switch between bottles more than once.

Comparison Among Species Among the three primates tested, the spider monkeys (with quinine hydrochloride,

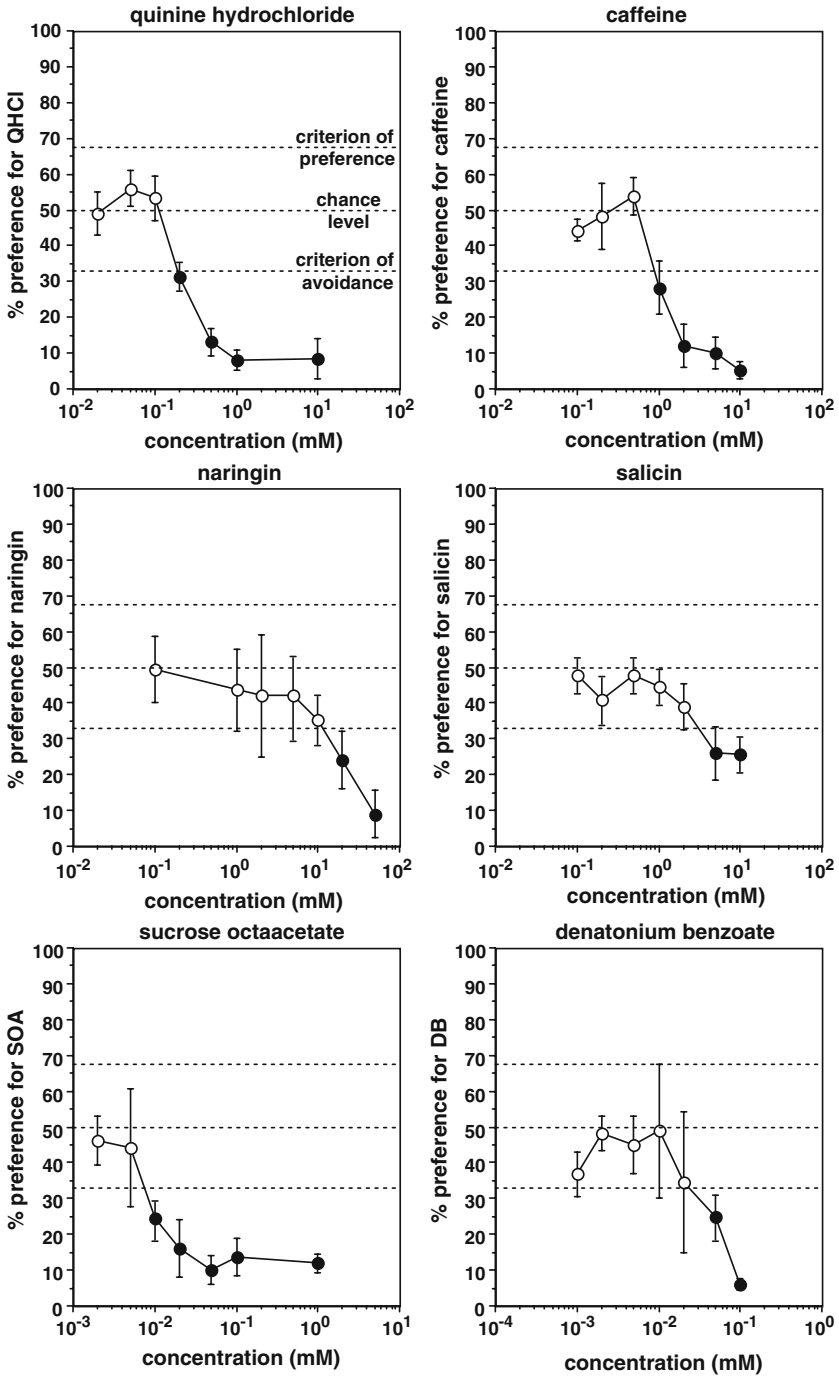

Fig. 1 Gustatory responsiveness of six squirrel monkeys to various concentrations of quinine hydrochloride, caffeine, naringin, salicin, sucrose octaacetate, and denatonium benzoate dissolved in a 30-mM sucrose solution and tested against a 30-mM sucrose solution as an alternative stimulus. Each data point represents the mean value (±SD) of ten test sessions of 2 min per animal. *Black* data points indicate concentrations at which all animals significantly rejected the bitter tastant (binomial test, $P<0.05$)

sucrose octaacetate, and denatonium benzoate) and the pigtail macaques (with caffeine, naringin, and salicin) showed the lowest taste avoidance thresholds with three of the six tastants each. The squirrel monkeys, in contrast, showed the highest taste avoidance thresholds with four of the six tastants (quinine hydrochloride, caffeine, naringin, and salicin). However, the across-tastant patterns of taste avoidance thresholds were identical between spider monkeys and squirrel monkeys, with both species displaying the following order of sensitivity: sucrose octaacetate > denatonium benzoate > quinine hydrochloride > caffeine > salicin > naringin. Accordingly, a significant positive correlation between the across-tastant patterns of sensitivity for the tastants tested with these two species was found (Spearman, $r_s=0.76$, $P<0.01$). The pigtail macaques displayed an across-tastant pattern of taste avoidance thresholds (sucrose octaacetate > caffeine > quinine hydrochloride > denatonium benzoate > salicin = naringin), which differed from that of the two other species. Accordingly, no significant correlation between the across-tastant pattern of *M.*

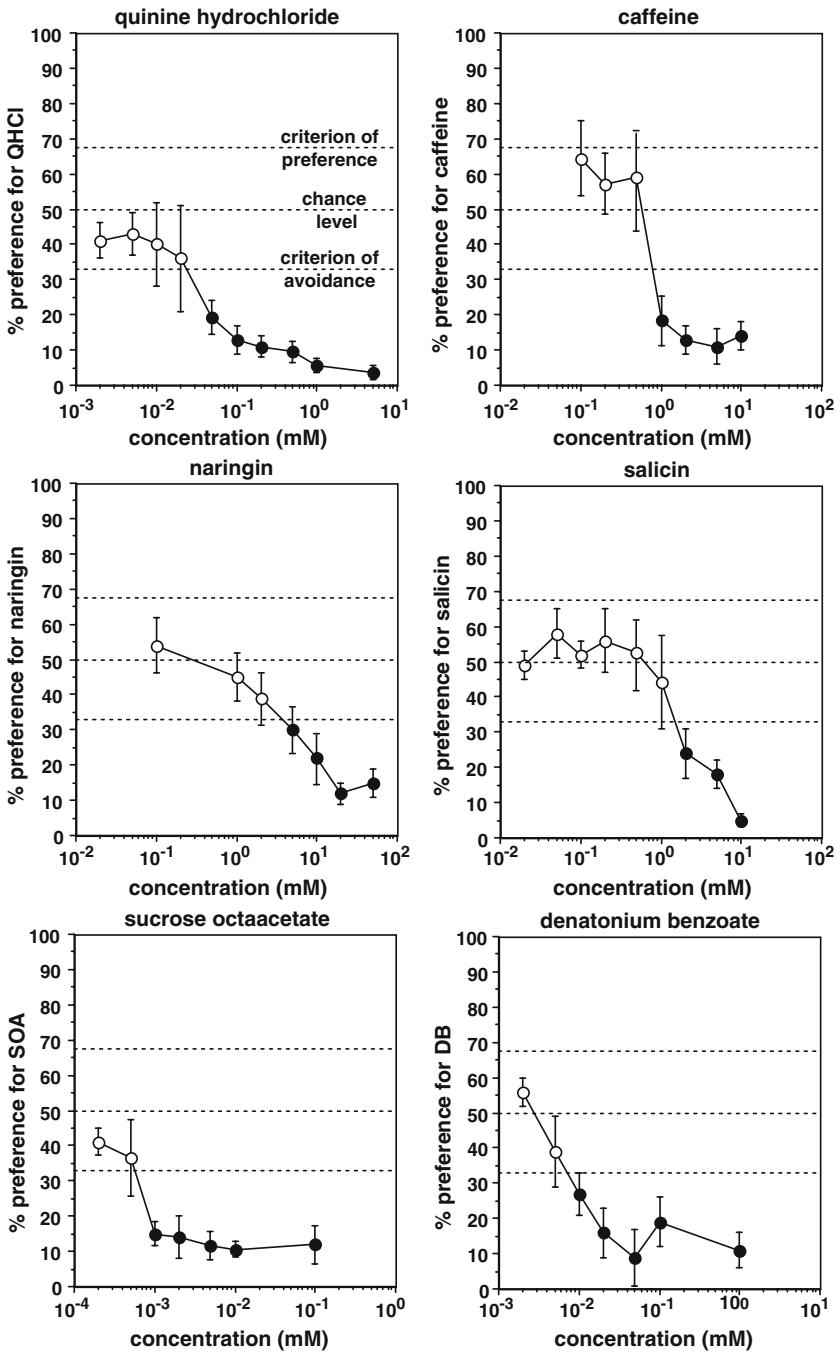

Fig. 2 Gustatory responsiveness of four spider monkeys to various concentrations of quinine hydrochloride, caffeine, naringin, salicin, sucrose octaacetate, and denatonium benzoate dissolved in a 30-mM sucrose solution and tested against a 30-mM sucrose solution as an alternative stimulus. Each data point represents the mean value (±SD) of ten test sessions of 2 min per animal. *Black* data points indicate concentrations at which all animals significantly rejected the bitter tastant (binomial test, $P<0.05$)

nemestrina and of *A. geoffroyi* (Spearman, $r_s=0.24$, $P>0.05$) and *S. sciureus* (Spearman, $r_s=0.26$, $P>0.05$) was found. Nevertheless, all primate species were most sensitive to sucrose octaacetate and least sensitive to naringin.

Discussion

The results of this study are a first and conservative approximation of the gustatory sensitivity of three primate species for the six bitter tastants tested. Although only between four and six animals per species were tested, the results appear robust as interindividual variability generally was low (see SDs in Figs. 1, 2, and 3). Further, with all tastants and all species, the lowest concentrations presented were consumed at equal amounts compared to the alternative stimulus, suggesting that the avoidance of (or, in the case of naringin with the pigtail macaques, preference for) higher concentrations of a stimulus was based on taste perception and not on other cues. However, the possibility of the tastants acting on olfactory and/or trigeminal receptors in the nose and/or the oral cavity cannot be

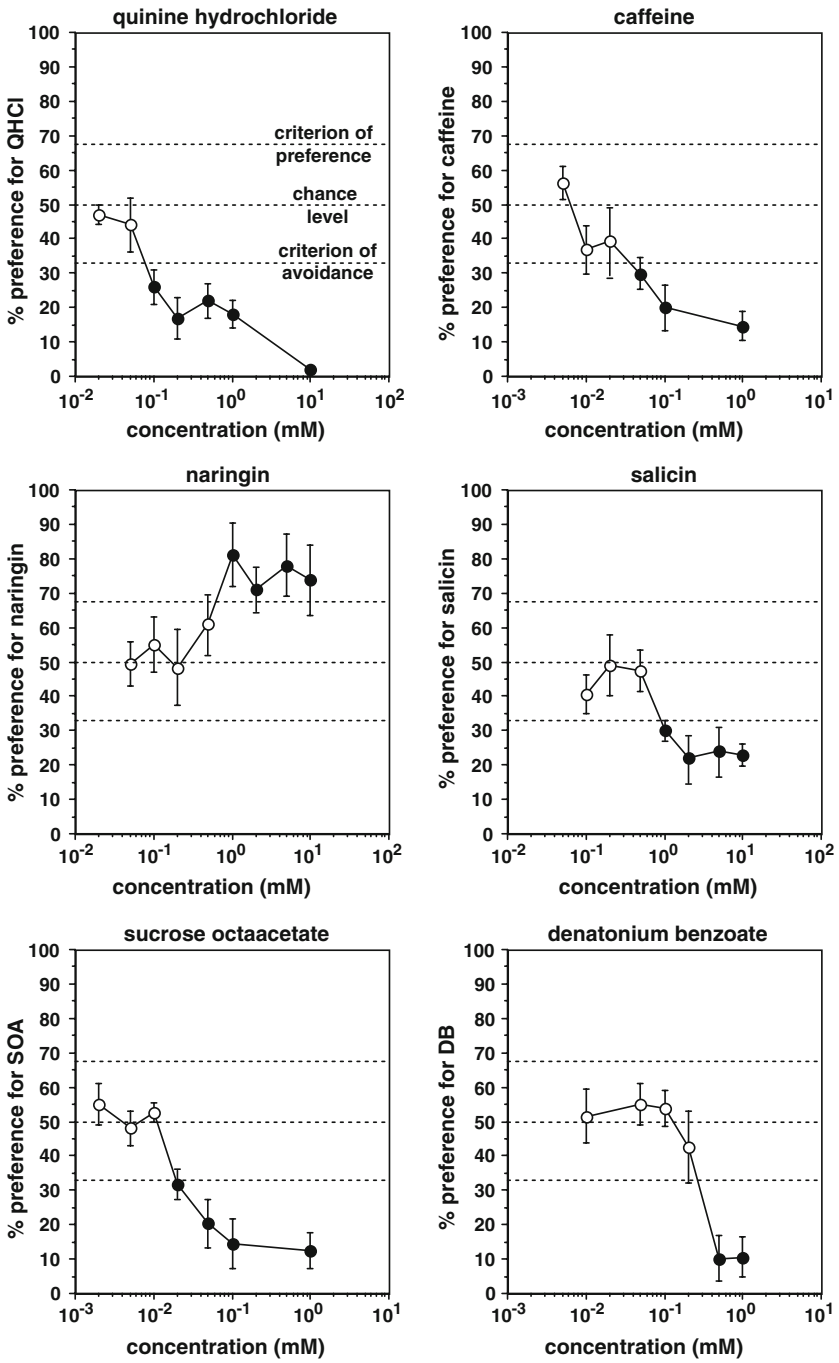

Fig. 3 Gustatory responsiveness of five pigtail macaques to various concentrations of quinine hydrochloride, caffeine, naringin, salicin, sucrose octaacetate, and denatonium benzoate dissolved in a 30-mM sucrose solution and tested against a 30-mM sucrose solution as an alternative stimulus. Each data point represents the mean value (±SD) of ten test sessions of 2 min per animal. *Black* data points indicate concentrations at which all animals significantly rejected (or, in the case of naringin, preferred) the bitter tastant (binomial test, $P<0.05$)

excluded. Similarly, the possibility that postingestive factors might have led to a conditioned aversion across trials that, in turn, might have affected the results cannot be excluded. There was no indication that the animals' avoidance of the bitter stimuli changed across trials. Taste qualities such as "bitter" are a human construct; it is not known whether other species experience the same percept as we do. Nevertheless, there is evidence of nonhuman primate taste receptors specialized to respond to and taste fibers, as well as cortical areas specialized to convey and process information about chemicals described as "bitter" by humans (Hellekant and Ninomiya 1994; Scott et al. 1999; Behrens and Meyerhof 2006).

Table 1 compares the taste avoidance thresholds of *S. sciureus*, *A. geoffroyi*, and *M. nemestrina* obtained here with data available from other species. Such across-species comparisons should take into consideration that different methods may lead to differing results. The present study employed a weak sucrose solution as solvent for the tastants, whereas other studies employed water. Similarly, in our study, the stimuli were presented for 2 min per trial, whereas some others have used different presentation times.

Additionally, the criterion used to define an avoidance threshold may differ among studies. All studies included in Table 1, however, employed two-bottle preference tests and were not conditioned to taste aversion or operant conditioning procedures and thus are basically the same method.

Whereas taste responsiveness to quinine hydrochloride has been tested with more than a dozen species of nonhuman primates and an even higher number of nonprimate mammals, data for the other tastants used in this study are sparse. The taste avoidance threshold of spider monkeys for quinine hydrochloride (0.05 mM) is one of the lowest values among the nonhuman primate species tested. It is interesting that the taste avoidance threshold of pigtail macaques for quinine hydrochloride (0.1 mM) is a factor 6–8 lower than that of the rhesus macaque and a factor of 10 lower than that of the long-tailed macaque, all species belonging to the same genus *Macaca*. All primate species tested here compare favorably in their responsiveness to quinine hydrochloride with the mouse. Other nonprimate mammals such as the cat (0.005 mM), the pig (0.002 mM), and the European mole (0.0005 mM) appear to be more sensitive for quinine hydrochloride than the primates tested here, whereas the horse (5.0 mM) and the rabbit (2.0 mM) show markedly higher avoidance threshold values and appear to be less sensitive than the primates tested here.

The only other data for any of the five other tastants used in the present study with other species of nonhuman primates were obtained with the rhesus macaque (Aspen et al. 1999). This Old World primate seems to be markedly less sensitive for caffeine (1.5 mM) than the pigtail macaque (0.05 mM) but similar to spider monkeys and squirrel monkeys (1.0 mM, respectively) and slightly more sensitive to denatonium benzoate (0.22 mM) than the pigtail macaque (0.5 mM), but less sensitive than the spider monkey (0.01 mM) and the squirrel monkey (0.05 mM).

The only species of nonprimate mammals for which data from all six tastants are at hand is the mouse. This rodent species appears to be less sensitive for caffeine and salicin and more sensitive for naringin than the three primates, but with avoidance threshold values for quinine hydrochloride, sucrose octaacetate, and denatonium benzoate falling into the range obtained with the three primate species. Although the across-tastant pattern of avoidance thresholds in the mouse (sucrose octaacetate > denatonium benzoate = quinine hydrochloride > naringin > caffeine = salicin) differs from those of the nonhuman primates tested, this species, too, appears to be more sensitive to the two artificial tastants compared to the four naturally occurring ones.

Our finding that the taste avoidance thresholds found here were about one (caffeine, naringin, and salicin) to four (denatonium benzoate) log units higher than the taste detection thresholds established in humans suggests that *Homo sapiens* is markedly more sensitive to bitter tastants than the primates tested here and, in fact, any nonhuman primate species tested so far (see Table 1). However, the sophisticated psychophysical signal detection methods employed with human subjects are likely to be more sensitive than the simple two-bottle preference test used with both nonhuman primates and nonprimate mammals. This assumption is supported by findings from Pritchard et al. (1995) who reported that taste thresholds obtained with a conditioning paradigm in *Macaca mulatta* are 1.6–2.2 log units lower than those obtained using a preference paradigm with the same species and are approximately 1.5 log units lower than human detection thresholds. This suggests that the sensitivity of the primates employed in our study might—at least for some of the tastants tested—be similar to or even match that of *H. sapiens*. The across-tastant pattern of taste detection thresholds obtained in humans (denatonium benzoate > sucrose octaacetate > quinine hydrochloride > caffeine > naringin > salicin) differs from those of the primates tested in our study, but humans, too, appear to be more sensitive to the two artificial bitter tastants compared to the naturally occurring ones.

Which factors might cause the differences in responsiveness within and between the species tested? It is well established that postingestive factors may influence an animal's ingestive behavior in subsequent encounters with the same type of food or liquid (Garcia and Hankins 1975). The method employed here, however, makes this possibility unlikely as the short duration of the tests (2 min per presentation) prevented animals from drinking amounts of liquid that are large enough to induce physiological effects that are perceptible.

Differences in dietary habits repeatedly have been shown to explain differences in taste performance among species (Kare 1971; Pfaffmann 1977; Spector 2000). Among New World primates, for example, the degree of frugivory correlates positively with sensitivity for food-associated monosaccharides and disaccharides (Laska 1996; Laska et al. 1996). Similarly, the proportion of animal matter in the diet of nonhuman primates correlates negatively with sensitivity for monosodium glutamate (Laska and Hernandez Salazar 2004). A comparison of 30 mammal species found a relationship between sensitivity for quinine hydrochloride and dietary specialization, with carnivores > omnivores > grazers > browsers (Glendinning 1994). This finding is consistent with the idea that animals with a relatively high occurrence of bitter and potentially toxic compounds in their diet, such as browsing herbivores, should have evolved a high bitter taste threshold (i.e., a low sensitivity) and, thus, a high tolerance to dietary toxins. In contrast, animals that rarely encounter bitter and potentially toxic compounds, such as carnivores, should have evolved a low bitter taste threshold (i.e., a high sensitivity) and, thus, a low tolerance to dietary toxins (Iason and Villalba 2006).

Table 1 Taste avoidance thresholds (in millimolar) for the six bitter tastants in the three primate species tested here (bold) and in other primates and nonprimate mammal species

species	QHCl	Caf	Nar	Sal	SOA	DB
Ateles geoffroyi	0.05	1.0	5.0	2.0	0.001	0.01
Saimiri sciureus	0.2	1.0	20	5.0	0.01	0.05
Macaca nemestrina	0.1	0.05	1.0	1.0	0.02	0.5
Homo sapiens	0.00097 (Glaser 1986)	0.002 (Schiffman et al. 1994)	0.22 (Schiffman et al. 1994)	0.2 (Bufe et al. 2002)	0.00036 (Schiffman et al. 1994)	0.0000042 (Schiffman et al. 1994)
	0.00048 (Schiffman et al. 1994)	3.2 (Smagghe and Louis-Sylvestre 1998)	0.14 (Smagghe and Louis-Sylvestre 1998)	0.2 (Soldo and Hofmann 2005)	0.004 (Boughter and Whitney 1993a, b)	
		1.0 (Soldo and Hofmann 2005)	0.14 (Drewnowksi et al. 1997)			
Macaca mulatta	0.63 (Glaser 1986)	1.5 (Aspen et al. 1999)				0.22 (Aspen et al. 1999)
	0.77 (Aspen et al. 1999)					
Macaca fascicularis	1.0 (Pritchard et al. 1994)					
Pan troglodytes	0.16 (Glaser 1986)					
Cheirogaleus medius	0.8 (Glaser 1986)					
Microcebus murinus	0.8 (Glaser 1986)					
	0.8 (Iaconelli and Simmen 2002)					
Loris tardigradus	0.8 (Glaser 1986)					
Nycticebus coucang	0.39 (Glaser 1986)					
Galago senegalensis	0.39 (Glaser 1986)					
Aotus trivirgatus	1.56 (Glaser 1986)					
Cebuella pygmaea	0.8 (Glaser 1986)					
	0.73 (Simmen and Hladik 1998)					
Saguinus midas niger	0.05 (Glaser 1986)					
Saguinus oedipus	0.065 (Simmen and Hladik 1998)					
Callithrix jacchus	0.53 (Simmen and Hladik 1998)					
Callithrix geoffroyi	0.37 (Simmen and Hladik 1998)					
Leontopithecus rosalia	0.21 (Simmen and Hladik 1998)					
Leontopithecus chrysomelas	0.055 (Simmen and Hladik 1998)					
Callimico goeldii	0.08 (Simmen and Hladik 1998)					

Nonprimate mammals						
Mus musculus	0.1 (Glendinning et al. 2002)	10 (Bachmanov, personal communication)	0.5 (Bachmanov, personal communication)	10 (Bachmanov, personal communication)	0.003 (Boughter et al. 2002)	0.1 (Whitney and Harder 1994)
					0.001 (Gannon and Whitney 1989)	0.2 (Boughter et al. 2005)
	0.2 (Boughter et al. 2005)				0.0001 (Harder et al. 1984)	0.1 (Bachmanov, personal communication)
					0.002 (Whitney and Harder 1994)	
Rattus norvegicus	0.27 (Brasser et al. 2005)	1.0 (Tordoff et al. 2008)			0.19 (Stewart et al. 1994)	0.55 (Brasser et al. 2005)
	0.02 (Cicala and McMichael 1964)					
	0.012 (Koh and Teitelbaum 1961)					0.032 (Tordoff et al. 2008)
	0.032 (Tordoff et al. 2008)					
	0.025 (Young et al. 1963)					
Clethrionomys glareolus	0.0093 (Chrzanowski 1965)					
Peromyscus melanotis	0.1 (Glendinning 1993)				1.0 (Glendinning 1993)	
Octodon degus	0.36 (Chrzanowski 1965)					
Mesocricetus auratus	2.0 (Carpenter 1956)	3.0 (Frank et al. 2004)			1.0 (Frank et al. 2004)	1.0 (Frank et al. 2004)
	0.3 (Frank et al. 2004)					
Cavia porcellus					1.0 (Jacobs 1978)	
Erethizon dorsatum	0.31 (Bloom et al. 1973)					
Felis silvestris catus	0.005 (Carpenter 1956)					
Oryctolagus cuniculus	2.0 (Carpenter 1956)					
Talpa europaea	0.0005 (Chrzanowski 1965)					
Crocidura russula	0.005 (Chrzanowski 1965)					
Erinaceus europaeus	0.1 (Ganchrow 1977)					
Odocoileus hemionus	0.13 (Crawford and Church 1971)					
Ovis aries	0.008 (Crawford and Church 1971)					
	0.48 (Goatcher and Church 1970a)					
	0.12 (Goatcher and Church 1970b)					
Sus scrofa	0.002 (Glaser et al. 2001)					0.1 (Nelson and Sanregret 1997)
Capra hircus	0.016 (Goatcher and Church 1970b)					
Bos taurus	0.06 (Goatcher and Church 1970b)					
Equus caballus	5.0 (Randall et al. 1978)					

QHCl quinine hydrochloride, *Caf* caffeine, *Nar* naringin, *Sal* salicin, *SOA* sucrose octaacetate, *DB* denatonium benzoate

This idea is supported further by the fact that browsing herbivores that cannot afford to reject all foods that taste bitter usually have evolved physiological mechanisms that aid in detoxifying potential toxins upon ingestion, while carnivores are usually lacking such evolutionary adaptations in their gastrointestinal tracts (Belovsky and Schmitz 1991).

The three species tested in this study differ markedly in the proportion of animal matter in their diet. However, squirrel monkeys, the species showing the highest taste avoidance thresholds with the naturally occurring tastants, also have been reported to show the highest proportion of animal matter in their diet (72% of total intake) among the three species tested. Pigtail macaques, the species with the lowest avoidance threshold with three of the four naturally occurring tastants, on the other hand, include a considerably lower proportion of animal matter in their diet (13%). Spider monkeys, with an intermediate position in their bitter taste responsiveness, hardly consume any animal matter (1% of total intake; Caldecott 1986; Lima and Ferrari 2003; Gonzalez-Zamora et al. 2009). Thus, our findings do not seem to support the presumed positive correlation between bitter taste sensitivity and carnivory. However, animal matter consumed by squirrel monkeys consists mainly of arthropods rather than vertebrates. Arthropods frequently employ bitter-tasting secretions or hemolymph as deterrents against predators (Eisner et al. 2005). The relatively low bitter taste sensitivity observed in squirrel monkeys might be explained as an evolutionary adaptation to bitter-tasting arthropod deterrents. This idea is supported by the fact that the pigtail macaques, which displayed lower avoidance thresholds for quinine hydrochloride than two other members of the genus *Macaca*, include a lower proportion of arthropods in their diet than rhesus and long-tailed macaques (Caldecott 1986).

Old World and New World primates differ in their ability to perceive substances such as aspartame and thaumatin, which both taste sweet to humans, suggesting that phylogenetic relatedness also might account for differences or correspondences in taste perception among species (Nofre et al. 1996). However, in our study, spider monkeys and pigtail macaques, i.e., one New World and one Old World primate species, displayed the lowest taste avoidance thresholds with three of the six tastants. The possibility of phylogenetic relatedness as an explanation for the observed differences in responsiveness to the bitter tastants tested is unlikely.

Pigtail macaques did not reject detectable concentrations of naringin but rather preferred them over the alternative stimulus. Although our use of a 30-mM sucrose solution, both as the solvent for the tastants and as the alternative stimulus, might have contributed to this unusual pattern of performance, this seems unlikely, as this concentration of sucrose is only a factor of 3 above this species' preference threshold (Laska 2000) and thought to be weak enough to prevent massive masking effects or other types of qualitative interactions that may occur when applied in heterogenous taste mixtures (Stevens 1996). A more likely explanation for the observed preference of the pigtail macaques for naringin is that this substance may not have a purely bitter taste for this species but may evoke a mixed taste sensation involving a quality that is not bitter. Human psychophysical studies (Schiffman 2000) as well as electrophysiological recordings from single taste fibers in nonhuman primates (Hellekant and Ninomiya 1994) have demonstrated that a variety of substances evoke mixed taste sensations and elicit activity in more than one type of taste fibers, i.e., not only in bitter-best fibers.

The concentrations detected by the three primate species with the four naturally occurring tastants are well below those reported in fruits or other parts of plants that are consumed by these species (Nagy and Shaw 1980; Souci et al. 1989; Rouseff 1990). However, bitter tastants may not be distributed uniformly in plants but may be concentrated in specific regions of fruits, seeds, or leaves. Nevertheless, it seems reasonable to assume that the bitterness elicited by these compounds in the natural diet of *S. sciureus*, *A. geoffroyi*, and *M. nemestrina* plays a part in the food selection behavior of these species.

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Cuticular Hydrocarbon Phenotypes Do not Indicate Cryptic Species in Fungus-Growing Termites (Isoptera: Macrotermitinae)

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Abstract In several termite species, distinct differences in the composition of cuticular hydrocarbons among colonies correspond to high genetic divergence of mitochondrial DNA sequences. These observations suggest that hydrocarbon phenotypes represent cryptic species. Different cuticular hydrocarbon phenotypes also are found among colonies of fungus-growing termites of the genus *Macrotermes*. To determine if these hydrocarbon differences in *Macrotermes* also indicate cryptic species, we sequenced the mitochondrial CO I gene from species in West and East Africa. Among individuals of a supposed species but belonging to different cuticular hydrocarbon phenotypes, the genetic distances are much smaller than distances between species. Unlike what has been observed in other termites, *Macrotermes* hydrocarbon phenotypes do not represent cryptic species. Our findings suggest fundamental differences in the evolution and/or function of cuticular hydrocarbons among different termite lineages.

Keywords Macrotermes · Termitidae · CO I mtDNA · Genetic divergence · Chemotaxonomy · Speciation · Cuticular lipids · Nestmate recognition · Kimura 2-parameter distance · Phylogenetics

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Introduction

Termites (Isoptera) have a complex social structure, similar to the social systems in some Hymenoptera, such as ants (Formicidae). However, these similar social structures are maintained through different intraspecific communication mechanisms. In ants, communication among nestmates is mediated by numerous glands and compounds (Hölldobler and Wilson 1990). In contrast, termites use a limited number of exocrine glands and few chemical compounds for communication (Billen and Morgan 1998; Kaib 2000; Peppuy et al. 2004). However, there is considerable evidence that termites use hydrocarbons on the epicuticle for nestmate and species recognition (e.g., Bagnères et al. 1991; Takahashi and Gassa 1995; Clément and Bagnères 1998; Kaib et al. 2002; Howard and Blomquist 2005). According to behavioral experiments, dissimilarity in the composition of cuticular hydrocarbons (CHC) are correlated with the level of agonistic behavior between colonies (e.g., Haverty and Thorne 1989; Haverty et al. 1999; Kaib et al. 2004a). Furthermore, inquilines, species from various insect orders that live within termite colonies, have a CHC composition similar to that of the host colony (Howard et al. 1980; Kaib et al. 2004b).

Insect CHCs range in chain length from C_{21} to C_{49} with saturated, unsaturated, and branched compounds (e.g., Lockey 1988; EstradaPena et al. 1994; Ye et al. 2007). This diversity allows for considerable qualitative and quantitative differences in the composition of CHCs among species or populations. Qualitative differences and large quantitative differences (CHC phenotypes) are interpreted to reveal variation among species, whereas moderate quantitative differences occur among conspecific populations (EstradaPena et al. 1994; Haverty and Nelson 1997; Takematsu and Yamaoka 1999; Page et al. 2002). Interspe-

cific differences in the composition of CHCs suggest that they are useful characters for species identification and taxonomy (Kaib et al. 1991; Bagine et al. 1994; Page et al. 2002; Ye et al. 2007). For example, in the termite genus *Reticulitermes* (Rhinotermitidae), CHC phenotypes correspond to mitochondrial DNA variation and provide evidence for cryptic species (Jenkins et al. 2000; Clément et al. 2001; Uva et al. 2004; Copren et al. 2005; Austin et al. 2007).

In several *Macrotermes* species, sympatric colonies have different CHC compositions (Bagine et al. 1994; Kaib et al. 2002, 2004a). The genus *Macrotermes* belongs to the fungus-growing Macrotermitinae within the most species-rich termite family Termitidae, also referred to as "higher termites". All other termite families are often labeled as "lower termites". To determine if qualitative CHC differences in higher termites also correspond to cryptic species, we sequenced the mitochondrial gene cytochrome *c* oxidase subunit I (CO I) from several *Macrotermes* species with differing CHC phenotypes.

Methods and Materials

We collected major workers from 27 colonies of *Macrotermes* from West and East Africa (Table 1) and divided the sample of each colony into two batches for chemical and genetic analysis, respectively. For each colony, we sequenced the CO I gene, a mitochondrial DNA fragment, which has been frequently used for the delimitation of taxonomic entities (Hebert et al. 2003; Smith et al. 2005; Aldrich and Kambhampati 2007). CHC composition was characterized for several of those colonies that were not previously analyzed in respect to DNA sequence variation (Kaib et al. 2002, 2004a). According to classical taxonomy, these colonies belong to four species. Colonies of the same

Table 1 *Macrotermes* samples used in this study and GenBank accession numbers for CO I gene sequences

Species name	Country	Location	Colony code[a]	CHC phenotype[a]	CO I haplotype	Accession number
M. falciger	Kenya	Kwale	F50	F I	1	FJ207434
M. falciger	Kenya	Kwale	F51	F II	2	FJ207435
M. falciger	Kenya	Kwale	F52	F II	3	FJ207436
M. falciger	Kenya	Kwale	F53	F II	2	FJ207437
M. falciger	Kenya	Kwale	F54a	F III	4	FJ207438
M. herus	Kenya	Kapenguria		na	5	FJ207439
M. herus	Kenya	south Nyanza		na	6	FJ207440
M. herus	Kenya	Kakamega		na	7	FJ207441
M. herus	Kenya	Nakuru		H I	8	FJ207442
M. herus	Kenya	Nakuru-Marigat		H II	9	FJ207443
M. jeanneli	Kenya	Marigat		na	10	FJ207449
M. jeanneli	Kenya	Kapenguria		na	10	FJ207450
M. subhyalinus[b]	Ivory Coast	Comoé	I1B1	S IV	11	FJ207424
M. subhyalinus	Ivory Coast	Comoé	I1B2	S IV	12	FJ207425
M. subhyalinus	Ivory Coast	Comoé	I1B3	S II	12	FJ207426
M. subhyalinus	Ivory Coast	Comoé	I2B1	S IV	13	FJ207427
M. subhyalinus	Ivory Coast	Comoé	I2B2	S I	12	FJ207428
M. subhyalinus	Ivory Coast	Comoé	I2B3	S IV	12	FJ207429
M. subhyalinus	Ivory Coast	Comoé	I1C3	S IV	12	FJ207430
M. subhyalinus	Ivory Coast	Comoé	I1C4	S I	12	FJ207431
M. subhyalinus	Ivory Coast	Comoé	I1D1	S II	12	FJ207432
M. subhyalinus	Ivory Coast	Comoé	I1D2	S III	13	FJ207433
M. subhyalinus[b]	Kenya	Namanga		na	14	FJ207444
M. subhyalinus	Kenya	Tsavo East		na	15	FJ207445
M. subhyalinus	Kenya	Magadi		na	16	FJ207446
M. subhyalinus	Kenya	Magadi		na	17	FJ207447
M. subhyalinus	Kenya	Magadi		na	18	FJ207448

[a] Chemical work has been documented previously, and we give the colony codes and phenotype labels used in the original publications (Kaib et al. 2002, 2004a)

[b] We treated *M. subhyalinus* from the Ivory Coast and from Kenya as separate species (for further details see "Methods and Materials")

Macrotermes species often build characteristic mounds. However, *Macrotermes subhyalinus* mounds differ considerably between West and East Africa. In the former, mounds are closed, whereas in the latter mounds have conspicuous open ventilation chimneys. Furthermore, cytochrome *c* oxidase subunit II gene sequences of *M. subhyalinus* suggest that these groups belong to two different species (Brandl et al. 2007). Nevertheless, we have retained the classic nomenclature for consistency with previous publications (Kaib et al. 2002, 2004a) but have treated *M. subhyalinus* from West and East Africa as different species in the present analysis (Table 1).

Chemical Analysis Chemical analysis of CHCs followed Kaib et al. (2002, 2004a) and most of the considered CHC phenotypes were previously documented there (see Table 1). In short, we extracted CHCs from up to ten major workers of each colony with *n*-hexane. Extracts were air-dried and reconstituted with *n*-hexane for analysis. After purification, the CHC compounds were separated by gas chromatography. Peaks from different chromatograms were aligned by comparison of linear retention indices calculated on the basis of an interspersed *n*-alkane series from eicosane to hexatriacontane. Individual hydrocarbons were identified by coupled gas chromatography/mass spectrometry. For the determination of the double bond positions of unsaturated hydrocarbons, dimethyl disulfide derivatives were prepared (see Kaib et al. 2000). For a descriptive analysis, we performed ordinations (e.g., principal component analysis based on variance–covariance matrices among individuals) to visualize the variation within and between colonies (see Kaib et al. 2002). In such ordination plots, individuals formed distinct groups, and in some species we also found distinct clusters of colonies. We identified these clusters with clear differences in the composition of CHCs as phenotypes. To characterize the composition of each phenotype, we averaged the relative concentration of each compound within colonies and across colonies of a particular phenotype (Fig. 1).

DNA Isolation, Amplification, and Sequencing We extracted DNA from the limbs of major workers by using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). A 658-bp fragment of the CO I gene was amplified with the primer pair HCO and LCO (Folmer et al. 1994) with a standard PCR protocol. Amplified DNA was purified by using the MinElute PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced in both directions by a commercial company (GENterprise GmbH, Mainz, Germany).

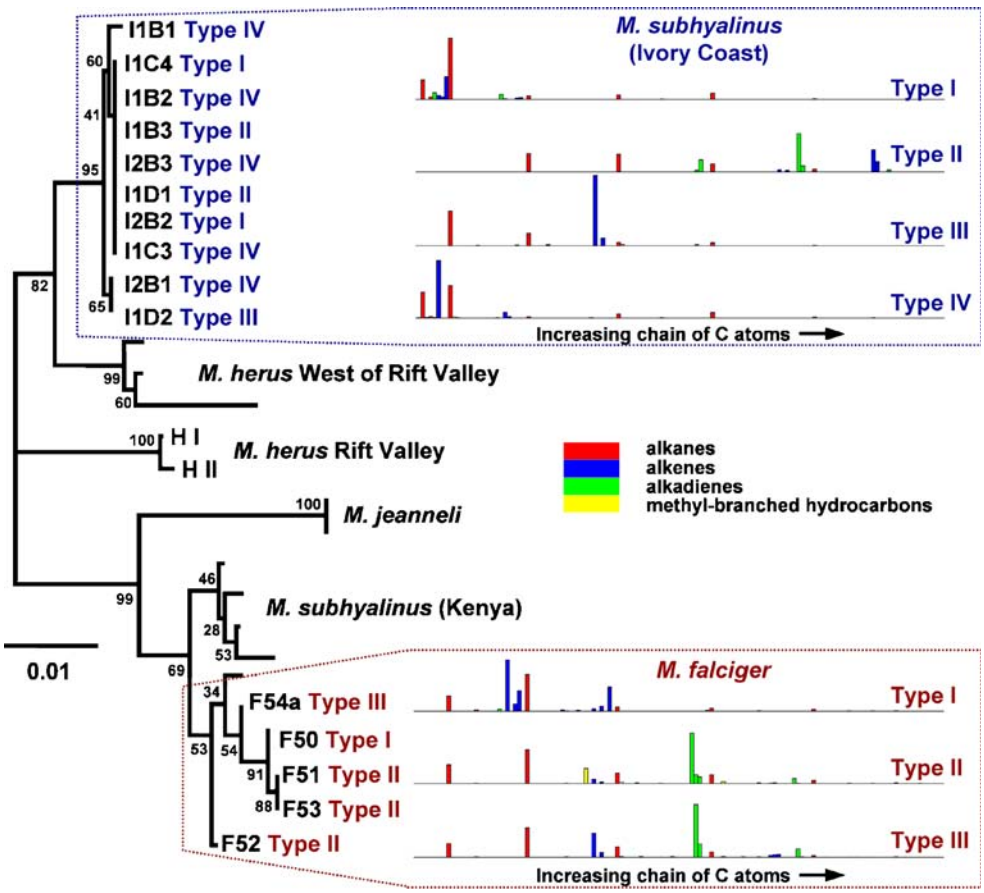

Fig. 1 Unrooted neighbor-joining tree of the CO I gene sequences obtained from *Macrotermes* colonies listed in Table 1 using the Kimura 2-parameter distance. Bootstrap support is shown at the nodes. For *M. subhyalinus* (Ivory Coast) and *M. falciger*, the relative composition of multiple CHC phenotypes (scale from 0% to 50%) is summarized. The legend indicates four different classes of hydrocarbons. For a detailed list of the compounds, see Kaib et al. (2002, 2004a)

Sequence Analysis The sequences were aligned with additional Macrotermitinae CO I sequences from GenBank (Supplementary material) by using ClustalW implemented in BioEdit 7.0.1 (Hall 1999) and visually checked. Kimura 2-parameter genetic distances among haplotypes were calculated in MEGA version 4 with the pairwise deletion option (Tamura et al. 2007). We accounted for the whole data set (own and published sequences) to calculate distances between certain CHC phenotypes, within species and between species pairs or genus pairs with the "within group means" and "between groups means" MEGA4 functions, respectively. The distance matrix for our own sequence data was used to construct a neighbor-joining tree, and support for the tree was evaluated with 1,000 bootstrap replicates. These simple methods were chosen for three reasons. First, we were interested in differences within and between congeneric species. The Kimura 2-parameter method increasingly underestimates distances with increasing differentiation. Therefore, our test is conservative for a comparison between distances within species (low downward bias) and distances between species or even higher taxa (high downward bias). Second, we were not attempting to determine the *Macrotermes* phylogeny. Instead, we were estimating the relative genetic differentiation between colonies with the same or different CHC phenotypes compared to the differentiation between species or genera. Finally, the Kimura 2-parameter distance required fewer parameters than other distance measures and had a lower associated error.

Results

Chemical Analysis The chemical composition differed little among major workers collected within the same colony (data not shown; for examples, see Kaib et al. 2002, 2004a). In addition, we showed for *Macrotermes herus* that minor and major workers have almost similar CHC profiles (data not shown). In contrast, in some cases CHC composition differed considerably among colonies of the same, morphologically defined species. For West African *M. subhyalinus*, four CHC phenotypes (Fig. 1) were identified (for further details see Kaib et al. 2004a). In contrast, East African *M. subhyalinus* showed no distinct phenotypes. In *Macrotermes falciger*, three unique CHC phenotypes were observed (Fig. 1, for further details see Kaib et al. 2002). Two *M. herus* colonies from the Rift Valley also had different phenotypes (data not shown). Within phenotypes, the variation of CHCs among colonies was small. Between colonies of a supposed species with different phenotypes, the composition of CHCs showed clear qualitative and quantitative differences, mainly in the unsaturated and branched compounds (Fig. 1).

Sequence Analysis We identified 62 variable sites in the CO I sequences. These sites resulted in 18 haplotypes among the 27 *Macrotermes* individuals (Table 1). The number of haplotypes per species ranged from 1 to 5. The most divergent haplotypes within a species differed by 21 transitions and seven transversions (haplotypes 6 and 9). These high values are due to differences between *M. herus* samples from the west of the Rift Valley in Kenya and samples within the Rift Valley (Fig. 1). The Kimura 2-parameter distance between the two groups was 0.035, whereas the distance within groups was 0.011 (west of the Rift Valley) and 0.0016 (within the Rift Valley; compare these values to Table 2). According to the CO I sequences, in Kenya *M. herus* may represent two different species, but the data are insufficient to draw conclusions. After excluding *M. herus*, haplotypes from the same species differed by at most three transitions and two transversions.

To determine the relationships among CHC phenotypes, we constructed a neighbor-joining tree (Fig. 1). Independent of CHC phenotypes, individuals of the same species clustered with high bootstrap support (>50%) in the tree. Different CHC phenotypes (e.g., phenotypes I, II, and IV of West African *M. subhyalinus*) even shared the same CO I haplotype (Fig. 1). Among the four categories where genetic distance was measured (among CHC phenotypes of a species, within species, between congeneric species,

Table 2 Kimura 2-parameter distances between CHC phenotypes of *Macrotermes* species, within species of Macrotermitinae, between congeneric species of Macrotermitinae, and between genera of Macrotermitinae

	Between phenotypes[a]	Within species[b]	Between species	Between genera
Minimum	0.0015	0.0011	0.0105	0.0791
Maximum	0.0031	0.0607	0.1306	0.1668
Mean	0.0016±0.0003	0.0229±0.0243	0.0874±0.0309	0.1392±0.0156
Number of comparisons	19	8	113	55

[a] CHC phenotypes of *M. falciger, M. herus*, and *M. subhyalinus* (Ivory Coast) were considered (see Table 1)

[b] To calculate distances within species, we used only sequences where no CHC data were available (own and published data, see also Supplementary material).

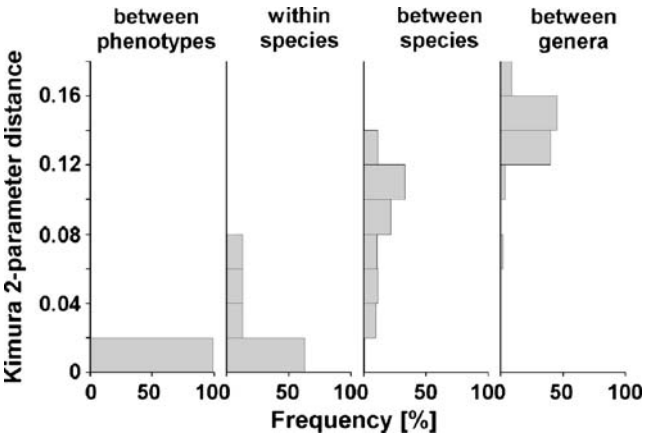

Fig. 2 Distribution of pairwise genetic distances among CHC phenotypes of *Macrotermes*, within species of Macrotermitinae, among congeneric species of Macrotermitinae, and between genera of Macrotermitinae (see also Table 2)

and between genera), distances between CHC phenotypes was the smallest (Fig. 2, Table 2). The maximum distance between conspecific CHC phenotypes (0.0031) was much smaller than the minimum distance between species (0.0105; Table 2).

Discussion

Based on the analysis of sequence variation of the CO I gene within and among species, CHC phenotypes in morphologically or geographically defined *Macrotermes* species do not represent cryptic species. This finding contrasts with what has been observed in lower termites. In lower termites, CHC phenotypes are supposed to represent cryptic species because of consistent differences in mitochondrial or nuclear DNA markers among phenotypes (e.g., Jenkins et al. 2000; Kutnik et al. 2004; Copren et al. 2005; Aldrich and Kambhampati 2007). CHC compositional differences within phenotypes are of similar magnitude in lower and higher termites (see Kaib et al. 2002, 2004a; Copren et al. 2005). Furthermore, genetic distances among species of Macrotermitinae are in the same range as those reported for lower termites (Park et al. 2006). Thus, fundamental differences in the evolution and function of cuticular hydrocarbons between lower and higher termites may exist.

We are aware that a single gene or locus on its own is not sufficient to detect cryptic species. However, the main goal of our study was to compare observations from lower termites with the situation in higher termites. Most of the studies on species specificity of CHC phenotypes in lower termites have used mitochondrial genes (Jenkins et al. 2000; Copren et al. 2005; Austin et al. 2007). Mitochondrial markers are sufficiently variable to resolve species-level differences, and such sequences also are valuable tools in termite taxonomy (Clément et al. 2001; Marini and Mantovani 2002; Szalanski et al. 2004; Uva et al. 2004; Park et al. 2006; Roy et al. 2006). For example, based on CO I gene differences, Aldrich and Kambhampati (2007) distinguished between subspecies of *Zootermopsis nevadensis* (Termopsidae). Furthermore, the CO I gene is an almost universal marker for the delimitation of species, and thus this gene is often used in barcoding studies (Hebert et al. 2003).

The suggested different role of CHC phenotypes in different termites is also exemplified by a difference in the geographic distribution of phenotypes. In lower termites, CHC phenotypes often occur in different populations (Haverty and Nelson 1997; Haverty et al. 1999; Page et al. 2002). Therefore, differences in CHC composition could be a passive consequence of other genetic differences or because of environmental differences. In contrast, in higher termites, CHC phenotypes may occur in the same population (Kaib et al. 2002, 2004a). Studies of CHC phenotypes in higher termites, however, are limited to the genus *Macrotermes*, and these patterns may not hold for all higher termites.

CHC compositional differences could be caused by genetic or environmental factors. In *Drosophila*, CHC composition is heritable (Takahashi et al. 2001). By using amplified fragment length polymorphism fingerprints, genetic differences in *M. subhyalinus* were correlated positively with differences in the composition of CHCs (Kaib et al. 2004a). Similar results, based on microsatellite data, were obtained for *Reticulitermes santonensis* (Dronnet et al. 2006). Although CHC composition may vary with environmental conditions, nesting material, and diet (e.g., Nielsen et al. 1999; Liang and Silverman 2000), even neighboring colonies may belong to different CHC phenotypes in *Macrotermes* (Kaib et al. 2002, 2004a). With such a small spatial distance among colonies, it is unlikely that environmental differences are the main factor for the occurrence of CHC phenotypes. Furthermore, *Macrotermes* species construct complex mounds with an effective ventilation system and constant microclimate conditions (Darlington 1985), and their CHC composition is not likely to reflect external environmental differences.

Regardless of the cause of CHC composition differences, interactions among workers from colonies with different phenotypes leads to aggression (Kaib et al. 2004a), indicating that CHCs play a role in nestmate recognition (Haverty et al. 1999; Kaib et al. 2002, 2004a, b). Nestmate recognition is essential to the social system because it guarantees colony integrity (see also Husseneder et al. 1998; Husseneder and Grace 2001). Because CHC composition is to some extent heritable and involved in nestmate recognition, divergent CHC composition could result in sympatric speciation, if alates use CHC for mate choice.

Alates are the colony members that swarm and mate with their counterparts from other colonies to establish new colonies. In *Macrotermes*, alates that establish new colonies are unrelated (Hacker et al. 2005). Because multiple CHC phenotypes occur in sympatry, there is a good chance that alates from different CHC phenotypes meet during nuptial flights. If alates are like workers, then CHC compositional differences among alates should lead to aggressive behavior. This aggression caused by different CHC phenotypes could lead to sympatric speciation. Reproductive individuals are often mutilated (Brandl et al. 2001, 2004), suggesting that aggressive behavior is common among reproductives, at least during the early stage of colony formation. However, our sequence data of the CO I gene suggest that the different CHC phenotypes do not represent distinct genetic lineages, and therefore CHC phenotypes are not mediating sympatric speciation. Rather than aggression as a mating barrier, in sympatric *Macrotermes* species, temporal separation of the nuptial flights and differences in sexual pheromones seem to prevent mating among closely related species (Peppuy et al. 2004).

Besides speciation, two other factors can explain the occurrence and evolution of CHC phenotypes in Macrotermitinae. First, CHC phenotypes may be influenced by symbionts. All *Macrotermes* species cultivate fungi in the colony for food (Pomeroy et al. 1991). Additionally, all termites harbor an abundant and diverse microbiota in their digestive tract (Brune 2006). If the fungi or the gut microbiota provide precursors for CHC synthesis, different fungal strains or differences in the composition of the gut microbiota could lead to different CHC profiles (Bagine et al. 1994). In this case, CHC phenotypes would result from fungal population structure or differences in the microbial gut community rather than from termite population structure. For example, it has been shown for the lower termite *Z. nevadensis*, that gut microorganisms supply precursors for methyl-branched hydrocarbon biosynthesis (Guo et al. 1991). In higher termites, however, a possible relationship between CHC composition and cultivated fungal strains or gut microbiota has never been tested. Second, the occurrence of CHC phenotypes might be influenced by inquilines. Termite colonies are long-lived and provide a rich, constant resource for inquilines. Colonies of fungus-growing termites often are invaded by termitophilous staphylinid or scarab beetles (e.g., Kistner 2001; Krikken 2008; own observations). If these inquilines become abundant, they can use up a significant portion of the food resources, thus lowering the fitness of the termite colony. Therefore, termites should have evolved strategies that limit colony access for inquilines. Inquilines have two possible strategies for access to the termite colony. Either inquilines are chemically neutral to the termites, or the inquilines acquire the recognition code of the colony. In support of the latter case, CHC composition of inquilines and termites was similar (Howard et al. 1980; Kaib et al. 2004b). However, this strategy would be risky for inquilines if different CHC phenotypes exist in a population of *Macrotermes* and inquilines have not evolved a fixed pattern of CHC composition that allows access to all colonies of a host species. Thus, the occurrence of different CHC phenotypes within populations of *Macrotermes* species might have evolved as a defense against inquilines.

Our COI gene sequence data suggest that CHC phenotypes do not represent species in *Macrotermes*. Therefore, in general, CHCs did not add new taxonomic information for species delimitation in this group (but see Kaib et al. 1991). It remains unknown if *Macrotermes* CHC composition is a consequence of the environment, symbiotic relationships, or if it is an evolutionary adaptation against unwanted visitors. Future studies need to investigate in *Macrotermes* the genetic population structure of symbionts as well as inquilines. Furthermore, we need more information on the genetic structure and CHC composition of other species of higher termites.

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Comparison of Urinary Scents of Two Related Mouse Species, *Mus spicilegus* and *Mus domesticus*

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Abstract Whereas the house mouse (*Mus domesticus*) has been studied extensively in terms of physiology/behavior and pheromonal attributes, the evolutionarily related mound-building mouse (*Mus spicilegus*) has received attention only recently due to its divergent behavioral traits related to olfaction. To date, no chemical studies on urinary volatile compounds have been performed on *M. spicilegus*. The rationale for our investigations was to determine if there are differences in urinary volatiles of intact and castrated *M. spicilegus* males and to explore further whether this species could utilize the same or structurally similar pheromones as the male house mouse, *M. domesticus.* The use of capillary gas chromatography/mass spectrometry (GC-MS) together with sorptive stir bar extraction sampling enabled quantitative comparisons between the intact and castrated *M. spicilegus* urinary profiles. Additionally, through GC-MS and atomic emission (sulfur-selective) detection, we identified qualitative molecular differences between intact *M. spicilegus* and *M. domesticus*. A series of volatile and odoriferous lactones and the presence of coumarin were the unique features of *M. spicilegus*, as was the notable absence of 2-sec-butyl-4,5-dihydrothiazole (a prominent *M. domesticus* male pheromone) and other sulfur-containing compounds. Castration of *M. spicilegus* males eliminated several substances, including δ-hexalactone and γ-octalactone, and substantially decreased additional compounds, suggesting their possible role in chemical communication. Some other *M. domesticus* pheromone components were also found in *M. spicilegus* urine. These comparative chemical analyses support the notion of metabolic similarities as well as the uniqueness of some volatiles for *M. spicilegus*, which may have a distinct physiological function in reproduction and behavior.

Keywords *Mus spicilegus* · *Mus domesticus* · Urinary volatile profile · Gas chromatography/mass spectrometry · Stir bar extraction · Pheromones

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Introduction

There is an evolutionary connection between the common house mouse (*Mus domesticus*) and the mound-building mouse (*Mus spicilegus)*, with two pairs of closely related species and subspecies in the phylogenetic tree: *Mus musculus domesticus* and *Mus musculus musculus* in one pair and *M. spicilegus* and *Mus macedonicus* in the second one (Bonhomme et al. 1984; Sage et al. 1993). The *M. domesticus* living environment is interwoven with human habitats, whereas *M. spicilegus* species is feral, and in some cases, at least during the summer period, *M. spicilegus* may live in a close contact with *M. musculus musculus* (Orsini et al. 1983; Sokolov et al. 1998; Simeonovska-Nikolova 2007). Additionally, in its social structure and behavior, *M. spicilegus* has diverged far from the *M. domesticus*. Unlike *M. domesticus*, *M. spicilegus* individuals are monogamous (Patris and Baudoin 1998; Dobson and Baudoin 2002; Baudoin et al. 2005; Gouat and Féron 2005) and cooperatively build colonial mounds for overwintering (Orsini et al. 1983; Garza et al. 1997; Poteaux et al. 2008).

Moreover, *M. spicilegus* males display intense paternal care (Patris and Baudoin 2000; Féron and Gouat 2007).

M. spicilegus individuals appear to rely on olfaction in their social behavior and mate selection (Patris and Baudoin 1998; Patris et al. 2002; Heth et al., 2001, 2003). In this species, social and kin recognition through olfactory cues have been observed in several studies (Gouat et al. 1998; Dobson and Baudoin 2002; Baudoin et al. 2005; Busquet and Baudoin 2005; Todrank et al. 2005; Colombelli-Negrel and Gouat 2006). The odors, which are used for social communication in mice, have a number of glandular and nonglandular sources: preputial glands, ear glands, plantar glands, anal glands, coagulating glands, submaxillary glands, urine, and feces (Brown 1985). The salivary androgen-binding proteins (Laukaitis et al. 1997; Talley et al. 2001) and tear fluid peptides (Kimoto et al. 2005) also have been reported as sources of chemical messengers in *M. domesticus*. In the house mouse, the urine-mediated chemical signals have been relatively well-characterized over the years (for reviews, see Novotny 2003; Hurst and Beynon 2004). Behavioral tests have revealed that the odor cues (expected to originate mainly from urine) are used in communication (Patris and Baudoin 1998; Féron and Gheusi 2003; Busquet and Baudoin 2005; Colombelli-Negrel and Gouat 2006). Thus far, there have been no complementary reports for *M. spicilegus* on the chemical nature of such olfactory cues; to date, the chemical constituents used in communication and scent sources for *M. spicilegus* remain unknown.

The first purpose of this study was to structurally characterize and quantify individual chemical constituents of the urinary volatile profiles for the intact versus castrated *M. spicilegus* males. The second goal was to qualitatively compare the findings to the previously well-characterized male *M. domesticus* urinary compounds.

A quantitative comparison of the intact and castrated *M. spicilegus* male volatile profiles was performed to explore the metabolic end products that could be produced under testosterone control. Some of the endocrinologically controlled urinary constituents may act as chemical messengers regulating various reproductive and social functions. Additionally, the urinary volatile profiles of female and male *M. spicilegus* individuals were qualitatively compared in order to explore gender roles related to the urine-mediated chemical communication, such as those used in mate selection and individual recognition. This chemical characterization is expected to provide clues to the observed differences in social and behavioral characteristics between the two species.

To facilitate this study, we used the stir bar aqueous extraction method (Baltussen et al. 1999, 2002), which was followed by solventless sample introduction into a gas chromatograph-mass spectrometer (GC-MS) instrument. This methodology is compatible with screening for volatile organic compounds at low concentrations in biological samples and is well suited for compound identification and quantitative comparisons (Soini et al. 2005). In addition, a combination of gas chromatography with atomic emission detection (GC-AED) was utilized for the highly sensitive sulfur compound profiling. In this report, we also take our previously determined characteristics of the male *M. domesticus* (ICR, C57Black/B6 and C57Black/B10) urinary volatile components and qualitatively compare them with the new *M. spicilegus* chemical information obtained in this study. Analytical approaches for the *M. domesticus* and *M. spicilegus* samples were identical. All analyses were performed in the same laboratory.

Methods and Materials

Experimental Animals M. spicilegus mice were fifth-generation animals from a population collected in Gyöngyös, Hungary in October 1999. From the time of collection, the genealogy of every individual was known, and all breeding pairs had been formed in a way to avoid inbreeding. They were bred at the University of Paris 13 (University of Paris-Nord at Villetaneuse) under laboratory conditions (20±1°C) with a 14:10 h L/D cycle. Food (mouse pellets type M20, Special Diet Services, Witham, Essex, UK), water, and bedding material (sawdust and cotton) were provided. Mice were weaned at 28 days of age and housed in same-sex sibling groups from 35 days of age. Males and females were 3–6 months old when they were used as urine donors.

For the male–female mouse comparisons, six males and six females were isolated in standard polycarbonate cages (26×16 cm and 14 cm high) 1 week before urine collection in order to eliminate the social dominance effect (Féron and Baudoin 1993, 1998). With the male presence being required to induce sexual receptivity in *M. spicilegus* females (Féron and Gheusi 2003), we assumed that all the isolated females were in anoestrus.

For the comparisons between intact and castrated males, isolation occurred 3 weeks prior to urine collection. Twenty males were isolated in standard polycarbonate cages (26× 16 cm, 14 cm high). Six of them were then castrated under anesthesia induced by an intraperitoneal injection of a mixture of ketamine (Imalgène 500, Merial, France, 100 mg/kg) and xylazine (Rompun 2%, Bayer, Puteaux, France, 5 mg/kg).

Male *M. domesticus* urinary volatile profiles from inbred ICR albino mice, C57BL /B6 and C57BL /B10 black mice (Jackson Laboratories, Bar Harbor, ME, USA) were used for qualitative comparisons. The data obtained for *M. domesticus* have been previously reported by Harvey et al. (1989) and Novotny et al. (2007).

Urine Collection Animals were introduced individually into a clean polycarbonate cage and surveyed for urination at least every 5 min. Fresh individual urine was quickly collected with a syringe and frozen (−20°C). A preliminary study showed that this was the best process to handle these sensitive mice.

Ethical Note The experiments complied with the current French laws (authorization 93-0033 for C. Féron; laboratory approval was secured from the Prefecture of Seine Saint Denis (prefectorial decree 02-2651), complying with the Association for the Study of Animal Behaviour/Animal Behaviour Society Guidelines for the Use of Animals in Research.

Reagents and Analytical Methods All compound identifications were verified by comparisons to authentic standards. Standard compounds were purchased from Aldrich Chemical Company (Milwaukee, WI, USA), except 3-octen-2-one and β-farnesene were from TCI America (Portland, OR, USA) or synthesized in our laboratory according to the previously described synthetic methods for dehydro-*exo*-brevicomin (Wiesler et al. 1984), *sec*-butyl-4,5-dihydrothiazole (North and Pattenden 1990), and 6-hydroxy-6-methyl-3-heptanone (Novotny et al. 1999). Twister™ stir bars (10 mm in length, 0.5 mm film thickness, 24-μl polydimethylsiloxane volume) were used as the sorptive extraction devices. They were purchased from Gerstel GmbH (Mülheim an der Ruhr, Germany). Volatile and semivolatile compounds were extracted from 0.2 ml of urine in 20-ml capped glass vials for 60 min with a Twister™ stir bar. The urine samples were first diluted with 2.0 ml water (high-purity OmniSolv® water, EM Science, Gibbstown, NJ, USA). As an internal standard, 8 ng of 7-tridecanone (Aldrich, Milwaukee, WI, USA) was added in 10 μl of ethanol to each vial. Stirring speed was 800+rpm on the Variomag Multipoint HP 15 stirplate (H+P Labortechnic, Oberschleissheim, Germany). Prior to extraction, all glassware were washed with acetone and dried at 80°C. After extraction, stir bars were rinsed with a small amount of distilled water, dried gently on a paper tissue, and placed in the Thermal Desorption Autosampler (TDSA) tube (Gerstel GmbH, Mülheim an der Ruhr, Germany) for the GC or GC-MS analysis. GC equipment for the sulfur compound analysis consisted of an Agilent GC Model 6890 instrument with an Atomic Emission Detector (Model G2350A from Agilent Technologies, Wilmington, DE, USA), and a Thermal Desorption Autosampler (Gerstel) operated in a splitless mode. The separation capillary column was HP-5MS (30 m×0.25 mm, i.d., 0.25 μm film thickness) from Agilent. Samples were thermally desorbed in a TDSA automated system, followed by injection into the column with a cooled injection assembly. Temperature program for desorption was 20°C (0.5 min), then 60°C/min to 280°C (hold 10 min). Temperature of the transfer line was set at 280°C. The injector was cooled with liquid nitrogen to −60°C and, after desorption and cryotrapping, was heated at 12°C/s to 280°C with the hold time of 10 min. The injector inlet was operated in the solvent-vent mode, with a vent pressure of 14 psi, a vent flow of 30 ml/min, and a purge flow of 50 ml/min. The GC temperature program was 40°C (0.5 min) then 2°C/min to 200°C (hold 10 min). The carrier gas head pressure was 14 psi for a flow rate of 1.2 ml/min. The GC unit was operated in the constant-flow mode. The emission lines for carbon (193 nm), sulfur (181 nm), and nitrogen (174 nm) were monitored during the atomic plasma emission detection.

The GC-MS instrument used for the compound identification was an Agilent 6890 N gas chromatograph connected to the 5973i MSD mass spectrometer (Agilent Technologies). The GC column was a narrow-bore capillary with 180 μm, i.d.×20 m DB-5MS (0.18 μm film thickness, Agilent Technologies, Wilmington, DE, USA). The inlet head pressure was 12.5 psi for the helium flow of 0.7 ml/min. The system operated in the constant-flow mode. The temperature program was 50°C (2 min) at 4°C /min to 200°C (hold 1 min). Positive electron ionization (70 eV) mode was used with the scanning rate of 4.51 scans per second over the mass range of 35–350 amu. The MSD transfer line temperature was set at 280°C. The ion source and quadrupole temperatures were set at 230°C and 150°C, respectively. The TDSA-injector sample introduction setting was identical to that described above in connection with the GC-AED system, except that the injector trapping temperature was set at −80°C.

Quantitative Evaluations and Statistical Analyses As the basis for quantitative comparisons of urinary chromatographic profiles of the fresh urine collected on plates, the peak area integration was performed, and peak areas were normalized by dividing with the peak area of internal standard (7-tridecanone) for each separated component. Either GC-MS total-ion chromatograms (TIC) or selected-ion chromatograms obtained after the postrun modification of TICs were used for calculations. Normalized peak areas were statistically evaluated for the intact and castrated mouse groups. Student's *t* test was employed for pairwise comparisons. The sulfur compound profiles from the GC-AED were compared in a qualitative manner between the intact and castrated males. The quantitative data obtained in this study for the male *M. spicilegus* urine samples were compared semiquantitatively with the female *M. spicilegus* urine and qualitatively with the male *M. domesticus* urine data obtained previously in this laboratory.

Results

Male *M. spicilegus* urinary volatile compound profiles by GC-MS featured more than 100 components. Approximately 60 compounds showed sufficient spectral intensity and

Table 1 Comparison of male *M. spicilegus* urinary compounds in intact (I) and castrated (C) samples

Compound	Retention time (min)	Comparison of levels (*I*) vs. (C)	Student's *t* test *P**	Intact mean ± SD (normalized peak area) *N*=14	Castrated mean ± SD (normalized peak area) *N*=6
5,5-Dimethyl-2-ethyl-4,5-dihydrofuran (DHF 1)	3.52	*I*>*C*	<0.001	464,084±224,499	51,261±29,647
Z-5,5-Dimethyl-2-ethylidenetetrahydrofuran (DHF 2)	4.97	*I*>*C*	<0.001	177,204±88,099	34,820±15,200
2-Heptanone	5.24	*C*=0		3,933,911±4,598,416	0
E-5,5-Dimethyl-2-ethylidenetetrahydrofuran (DHF 3)	5.72	*I*>*C*	0.002	76,468±43,006	12,070±6,267
p-Cymene	9.13	*I*>*C*	0.028	5,367,885±1,995,779	3,344,710±699,330
3-Octen-2-one	9.60		>0.05	89,763±80,251	146,340±152,691
Dehydro-*exo*-brevicomin (DHB)	9.93		>0.05	340,327±351,343	104,202±123,621
γ-Hexalactone[b]	9.99	*I*>*C*	0.023	4,535,674±2,436,519	1,998,715±592,374
Acetophenone	10.46	*I*>*C*	0.013	525,485±435,655	30,341±12,777
o-Toluidine	10.61		>0.05	1,441,690±1,919,224	37,787±37,458
δ-Hexalactone[b]	11.37	*C*=0		426,477±357,873	0
Undecane	11.76	*I*>*C*	0.01	2,155,122±756,483	1,123,143±676,228
Nonanal	11.91	*I*>*C*	0.018	3,688,456±1,621,797	1,921,820±247,488
Phenylacetone[a]	12.55		>0.05	1,406,846±1,805,201	136,475±227,297
A lactone[b,a]	13.38	*I*>*C*	0.001	312,203±110,744	83,383±34,966
Undecalactone[a,b]	13.63	*I*>*C*	0.032	218,191±177,650	45,713±28,089
4-Ethylphenol[b]	13.95	*I*>*C*	0.003	298,778±91,715	135,636±116,851
A methyl toluate[a,b]	14.28		>0.05	653,578±453,522	4,109,225±6,503,658
Octanoic acid	14.39		>0.05	304,124±281,096	122,047±133,034
Decanal	15.42		>0.05	1,647,466±1,005,640	860,987±342,581
Unidentified m/z 121[b]	15.63	*I*=0		0	373,429±196,797
N-Phenylformanilide[a]	15.65	*C*=0		4,635,124±5,249,321	0
Unidentified m/z 140[b]	15.83	*C*=0		431,407±265,044	0
2-Coumaranone[b]	16.24	C>I	<0.001	3,979,595±1,610,512	44,592,400±32,762,900
A lactone[a,b]	16.96	*I*=0		0	679,252±326,535
γ-Octalactone[b]	17.16	*C*=0		341,615±179,053	0
A lactone[a,b]	17.29		>0.05	454,554±185,246	901,687±977,437
Nonanoic acid	17.64		>0.05	120,761±90,627	1,099,975±2,101,058
Decanol	17.68		>0.05	2,025,997±1,869,935	738,656±643,914
δ-Nonyl-δ-valerolactone[a,b]	17.82		>0.05	922,899±2,019,056	55,588±21,976
A ketone[a]	17.9	*I*>*C*	0.036	6,645,912±5,406,927	1,531,829±749,722
Indole	18.16		>0.05	621,379±681,142	320,436±253,647
Coumarin[b]	18.35	*I*=0		0	285,687±253,621
γ-Nonenolactone[b]	19.77	*C*>*I*	0.048	419,497±193,279	928,870±878,601
A lactone[a,b]	20.45	*I*=0		0	1,314,727±712,105
An acid[a,b]	20.90		>0.05	254,023±156,938	528,732±722,600
Undecanol	21.05		>0.05	4,985,273±3,841,959	1,594,441±1,155,482
N-(Methylthio)methylaniline[a]	21.92	*I*>*C*	0.01	2,068,105±1,468,134	288,766±414,582
Dodecanal	22.15		>0.05	2,108,451±1,246,395	1,308,000±463,597
Geranylacetone	23.31		>0.05	1,037,403±651,760	898,834±1,411,928
β-Farnesene	23.47	*C*=0		313,220±357,030	0
m/z 125 δ-Lactone[a,b]	23.68	*I*>*C*	0.003	366,068±177,288	97,930±89,840
Unknown[b]	23.78	*I*=0		0	157,963±107,125
Unknown[b]	23.97	*I*=0		0	562,598±243,505
Dodecanol	24.19	*I*>*C*	0.028	12,682,500±7,735,907	4,775,774±2,980,101

Table 1 (continued)

Compound	Retention time (min)	Comparison of levels (*I*) vs. (C)	Student's *t* test *P**	Intact mean ± SD (normalized peak area) *N*=14	Castrated mean ± SD (normalized peak area) *N*=6
Unknown[b]	24.99		>0.05	1,587,800±1,972,417	53,771±131,711
Unknown[b]	25.51	*I*=0		0	3,411,073±1,893,080
Dodecanoic acid	26.87		>0.05	988,836±1,058,383	451,964±413,834
An acid[a,b]	27.34	*I*=0		0	8,786,688±3,876,541
Unknown[b]	33.66	*I*=0		0	8,514,168±3,430,211
N,*N*-Dimethyldodecanamide[a,b]	34.30		>0.05	9,643,776±4,582,043	6,694,015±6,896,710
Unknown[b]	34.69	*I*=0		0	3,201,660±2,890,689
Palmitic acid	37.37	*I*>*C*	0.042	2,142,529±1,710,270	585,610±167,857
Unknown[b]	38.30	*I*=0		0	2,264,620±793,599
Unknown[b]	38.82	*I*=0		0	13,472,067±3,126,728

*$P<0.05$ accepted significance; statistical significance for the compound level differences between intact (*I*) and castrated (*C*) male *M. spicilegus* urine

[a] Tentatively identified

[b] Unique urinary compounds for *M. spicilegus*, not found in *M. domesticus*

purity for the quantitative comparisons. Among these, 30 were identified, 15 were tentatively identified, while ten remain unknown (Table 1).

The characteristic feature for the profiles from *M. spicilegus* was the prominent presence of γ- and δ-lactones, ketones, alcohols, and acids. Characteristic urinary components found in *M. domesticus* are three dihydrofuran compounds (MW 126), which were shown previously to originate from a puberty-accelerating pheromone, 6-hydroxy-6-methyl-3-heptanone (Novotny et al. 1999) and its lactol form (see Table 1 for 5,5-dimethyl-2-ethyl-4,5-dihydrofuran, *Z*-5,5-dimethyl-2-ethylidenetetrahydrofuran and *E*-5,5-dimethyl-2-ethylidenetetrahydrofuran). These three furan derivatives were present in male *M. spicilegus* at levels comparable to those for *M. domesticus* reported earlier (Harvey et al. 1989; Novotny et al. 2007). In castrated *M. spicilegus*, the levels of these furan derivatives were significantly lower ($P<0.002$) than in intact *M. spicilegus* males. Another *M. domesticus* pheromone compound, dehydro-*exo*-brevicomin, also present in female urine in small amounts (Harvey et al. 1989; Jemiolo et al. 1991), was found at lower levels in urine from the intact and castrated male and also in female *M. spicilegus* urine (data not shown). Castration did not change dehydro-*exo*-brevicomin levels in *M. spicilegus* significantly in contrast to the suppressed levels of dehydro-*exo*-brevicomin in the samples from castrated *M. domesticus* (Novotny et al. 1980; Harvey et al. 1989). Trace levels of the dominant male mouse pheromone for *M. domesticus*, β-farnesene (Harvey et al. 1989), were detected in the intact male *M. spicilegus* urine but were not seen in female or castrated animals. *N*-(Methylthio) methylaniline was the only identified sulfur compound in the GC-MS TIC urine profiles from *M. spicilegus*, being more abundant in the intact male mouse urine when compared to the mouse urine of castrates ($P<0.01$). The representative structures of the main compounds distinguishing the two species are shown in Fig. 1.

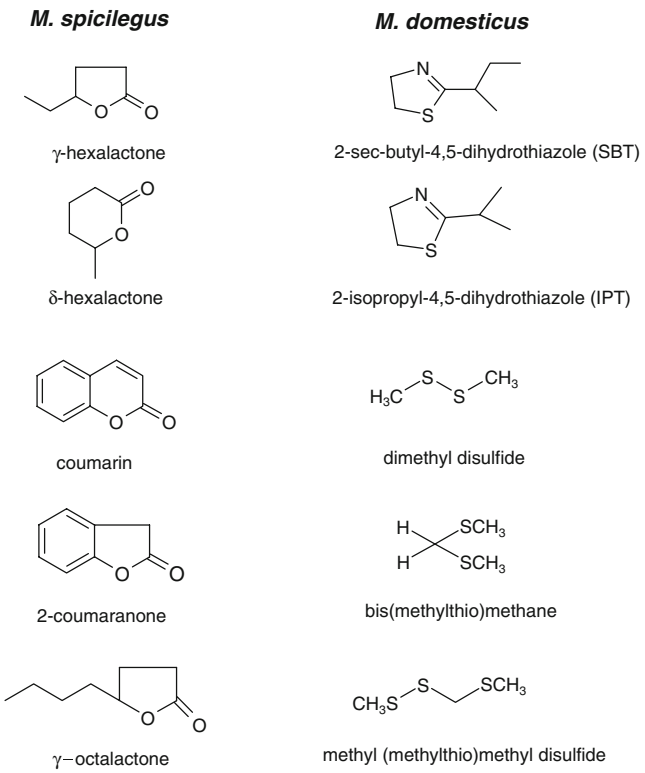

Fig. 1 Distinguishing chemical structures of volatile compounds in *M. spicilegus* and *M. domesticus* male urine

Castration of *M. spicilegus* males affected the volatile profiles in three ways: certain compounds, apparently under endocrine control, disappeared; while the levels of other compounds increased; and a set of previously undetected compounds became apparent (see Table 1). Figure 2 illustrates the representative urinary volatile profiles for intact and castrated *M. spicilegus* males and intact male *M. domesticus*. In addition to β-farnesene, castration removed 2-heptanone, δ-hexalactone, *N*-phenylformanilide, and γ-octalactone from the set of urinary volatiles. Some compound levels decreased after castration, including γ-hexalactone ($P<0.02$) and *N*-(methylthio)methylaniline ($P<0.01$), shown as normalized peak areas with standard deviation (SD) in Table 1. In contrast, after castration, levels of 2-coumaranone, a unique compound for *M. spicilegus*, increased ($P<0.001$). The absolute amounts of 2-coumaranone in castrated male urine were about 300± 200 ng/ml (standard deviation, SD, $N=6$), while in the intact mouse urine, the levels were just 2±2 ng/ml (SD, $N=14$). In addition, other constituents, including several lactones, were found at higher levels, and new compounds such as coumarin and several late-eluting lactones appeared in the urine of castrated males (see Table 1). Figure 3 shows a comparison of selected compound levels affected by castration. Individual variation for some of the compounds appeared relatively large (>70%, relative standard deviation (RSD), $N=6$–14), while some compounds varied only within the range of 12–30% (RSD, $N=6$–14) among the sampled individuals. Typically, the variation due to the sampling method was only 5–10% (RSD, $N=4$).

Qualitative comparisons in the GC-AED sulfur-selective profiles did not reveal any clear differences between intact

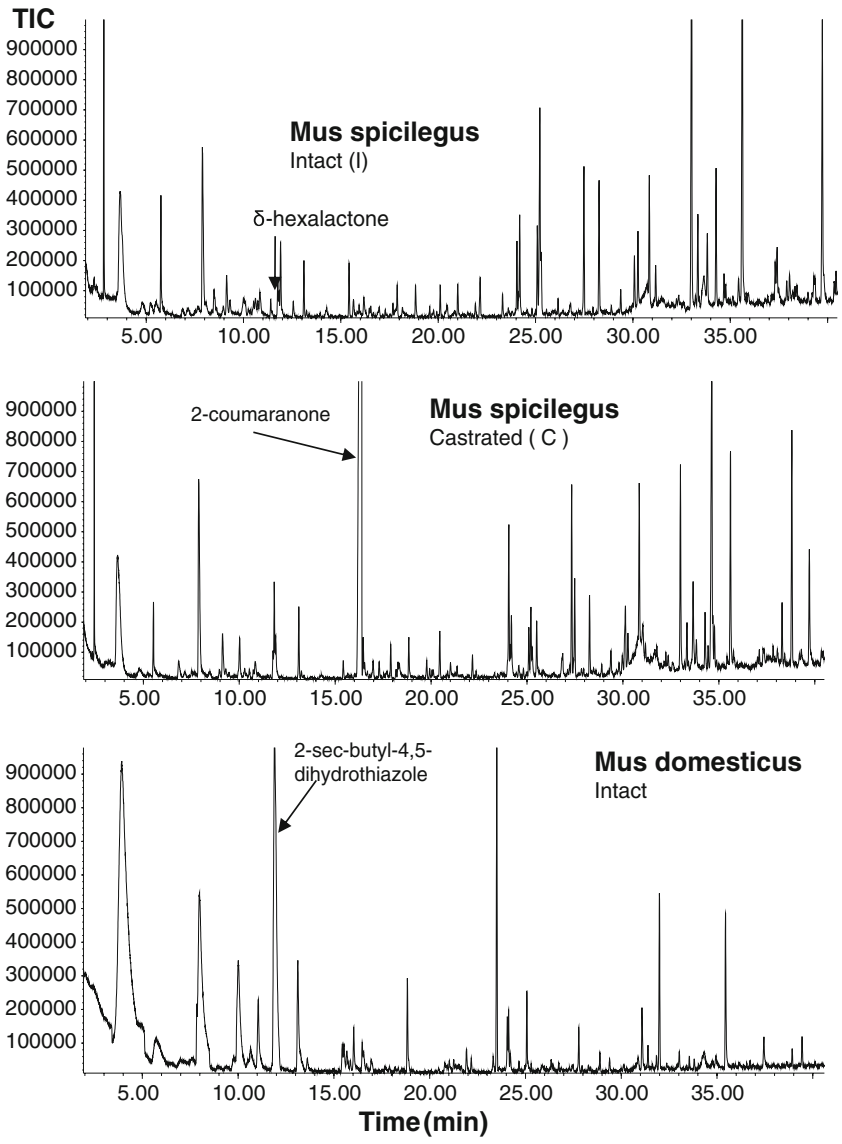

Fig. 2 Representative urinary (GC-MS total ion current) profiles of **a** intact and **b** castrated male *M. spicilegus*. **c** A comparative profile for male intact *M. domesticus*

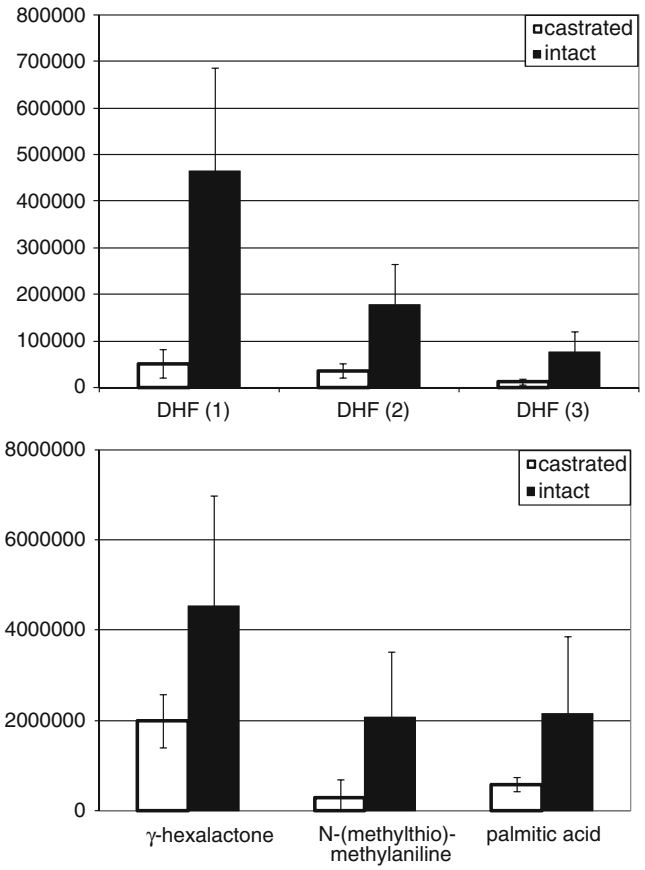

Fig. 3 Effect of castration on selected compound levels in urine (see Table 1). *Y-axis* corresponds to normalized peak areas. *Error bars* indicate standard deviation (SD)

and castrated animals. Relatively low-level (sub-picogram), sulfur-containing compounds (data not shown) were detected but could not be identified structurally.

Several of the previously reported *M. domesticus* urinary compounds (Novotny et al. 1990a, b, 2007) also were present in *M. spicilegus* profiles, including three characteristic dihydrofurans. It is notable that the *M. domesticus* male dominance signaling pheromone compound, 2-sec-butyl-4,5-dihydrothiazole, and its "structural relative," 2-isopropyl-4,5-dihydrothiazole (Novotny et al. 1985), were not detected in the *M. spicilegus* urine. Linear sulfur compounds, such as dimethyl disulfide, bis(methylthio) methane, and methyl(methylthio)methyl disulfide, typical for *M. domesticus* (Novotny et al. 2007), were also absent in *M. spicilegus* urinary volatile profiles. Furthermore, few *M. spicilegus* urinary ketones were present compared to those identified in *M. domesticus* urine (Novotny et al. 2007).

Discussion

Since castrated male *M. spicilegus* did not show β-farnesene, 2-heptanone, δ-hexalactone, *N*-phenylformanilide, or γ-octalactone among the urinary volatiles, it is suggested that the metabolic pathways involving these compounds may be under endocrine control. Behavioral tests with these compounds would be necessary to show what are the possible chemo-signaling and physiological functions of these compounds for male and female *M. spicilegus.* Castration also significantly affected the production of lactones, thus demonstrating an endocrine feedback for lactone biosynthesis. There is a strong possibility that some of the lactones may be mediators of chemical communication that involve reproduction. Furthermore, lactone levels were particularly varied among the individual intact males, suggesting that the lactone profile could be related to individual recognition. In their pure form, many of the lactones exhibit fruit- or berry-like aromas (Gatfield et al. 1993), which are relatively subtle odors in human perception, as opposed to the pungent smell in the *M. domesticus* male urine caused by the sulfur-containing compounds. The same unique lactone compounds were found in the female *M. spicilegus* urine in our qualitative screening (unpublished experiments).

Lactone biosynthesis involves C-18 hydroxyl fatty acids as precursors, which undergo β-oxidation steps followed by lactonization (Albrecht et al. 1992). Additionally, 9,10-oleic acid has been reported as a precursor for γ-dodecalactones in the yeast cultures (Haffner and Tressl 1996). This may imply that the metabolism of fatty acids leading to urinary lactone end products could play a prominent role in *M. spicilegus* metabolism. Contrarily, *M. domesticus* shows little presence of urinary lactones. The occurrence of urinary lactones previously has been reported in female and male pine voles (*Microtus pinetorum*) (Boyer et al. 1989). In female pine voles, γ-octalactone exhibited the greatest urinary level changes among the volatile compounds after estrogen treatment or ovariectomy.

Other biological sources for lactones have been reported for insects and microorganisms, such as the cephalic gland γ-octalactone of giant honeybee workers (*Apis laboriosa*; Blum et al. 2000) and δ- and γ-lactones emitted by marine *Alphaproteobacteria* (Dickschat et al. 2005). In these two studies, lactones were hypothesized as potential chemical signaling compounds within the giant honeybee colony and bacterial culture, respectively.

Urinary ketones in intact male *M. spicilegus* interestingly were sparse (Table 1). *M. domesticus* urinary ketones (e.g., 2-heptanone, 6-methyl-5-hepten-3-one, 5-hepten-2-one) previously have been found to correlate with the major histocompatibility complex mouse haplotypes (Novotny et al. 2007).

M. spicilegus originate from Eastern Europe. Several diagnostic genetic loci separate *M. spicilegus* and *M. domesticus* species (Bonhomme et al. 1984; reviewed in Sokolov et al. 1998). The genes in these loci code for enzymatically active proteins. Alcohol and malate dehy-

drogenases, esterases, carbonic anhydrase, and mannose and glucose phosphate isomerases are among these proteins. Consequently, some of the distinguishing coded enzymes may impact the metabolic pathways that lead to the excretion of urinary substances.

In addition to the lactones, urinary coumarin appears as an interesting "metabolic marker compound" for the *M. spicilegus* species. The presence of coumarin and abundance of 2-coumaranone in the urine of castrated *M. spicilegus* male also represent distinguishing metabolic pathways not found in *M. domesticus*. Coumarin could originate from different plants (reviewed in Bourgaud et al. 2006). In mammalian systems, coumarin generally exhibits toxic effects and is oxidatively metabolized (detoxified) by the cytochrome P450 mono-oxygenase enzyme system in liver microsomes (Creaven et al. 1965; Lewis and Lake 2002). The specific mouse enzyme for coumarin elimination through 7-hydroxylation is CYP2A5 (Miles et al. 1990). The CYP2A5 enzyme is also known to be inhibited by lactones and 2-coumaranone (Juvonen et al. 1991, 2000). In a study within the *M. domesticus* strains, P450 enzymes have been found genetically altered among these strains (Wood 1979). Furthermore, a single autosomal gene locus Gpi-1 (glucose phosphate isomerase-1) was found responsible for the differential hydroxylase activity of P450 (Wood and Taylor 1979). Since Gpi-1 also was found as one of the distinguishing genetic loci between *M. spicilegus* and *M. domesticus* (Bonhomme et al. 1984), the vastly different urinary volatile profiles of these species could be due, in part, to differential P450 oxidase activity, among other metabolic routes. For example, the distinguishing Es-2 loci controlling esterases have been found especially active in the kidney (Ruddle et al. 1969) and thus likely to affect some of the urinary metabolite excretion. However, separate genetic studies and metabolic mapping are necessary to link the genetic sources for the observed metabolic profile differences between *M. spicilegus* and *M. domesticus*, as exemplified by the urinary coumarin levels observed in this study. Furthermore, it seems desirable to investigate how these genetically induced changes in the urinary volatile constituents could facilitate chemical communication and social behavior in the "scent world" of the *M. spicilegus* species.

In summary, quantitative comparisons of the urinary volatile profiles for male *M. spicilegus* mice reveal several compounds that have previously shown biological activity as components of male-produced pheromones in the *M. domesticus* species. These similarities suggest that the two mouse species carry a certain genetic linkage that may be utilized in chemo-signaling. On the other hand, the total absence of the prominent *M. domesticus* male aggression pheromone, 2-*sec*-butyl-4,5-dihydrothiazole, in the *M. spicilegus* urine and the presence of unique δ- and γ-lactones and coumarin seem to indicate that these species have developed some distinctly separate metabolic pathways involving urinary constituents. Castration of *M. spicilegus* males removed δ-hexalactone and γ-octalactone among the identified urinary constituents. Their testosterone control suggests a possible involvement in chemical communication within the species. Behavioral tests are in progress to define possible roles of several urinary volatile organic compounds in *M. spicilegus* chemical communication.

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Identification and Synthesis of a Female-Produced Sex Pheromone for the Cerambycid Beetle *Prionus californicus*

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Abstract Females of the large cerambycid beetle *Prionus californicus* produce a powerful sex pheromone that attracts males. The pheromone was adsorbed on solid phase microextraction (SPME) fibers inserted into the ovipositor sheath and analyzed by coupled gas chromatography-electroantennogram detection and GC-mass spectrometry. The pheromone was identified as an isomer of 3,5-dimethyldodecanoic acid by a combination of retention index comparisons and mass spectral interpretation. The mass spectrum was misleading because it exhibited enlarged fragment ions that were not representative of branch points or other obvious stabilizing structural elements. The structure was verified by synthesis of 3,5-dimethyldodecanoic acid as a mixture of all four possible isomers, and this mixture was highly attractive to male beetles in field bioassays. The SPME extracts also contained several other compounds that were tentatively identified as chain-extended homologs of the main pheromone component. This pheromone should prove useful for sampling and management of the beetle, which is an important pest of hops, and an occasional pest in a variety of orchard crops. Although this is the first female-produced pheromone to be identified from the Cerambycidae, there is considerable evidence for pheromone production by females of other species in the subfamily Prioninae. Thus, this pheromone and the associated methodology used in its identification should be useful in the identification of female-produced attractant pheromones from other members of the subfamily.

Keywords 3,5-dimethyldodecanoic acid · 3,5-dimethyltridecanoic acid · 3,5-dimethylpentadecanoic acid · Cerambycidae · Prioninae · Coleoptera

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Introduction

Prionus californicus Motschulsky (Coleoptera: Cerambycidae), commonly known as the California prionus, ranges throughout much of western North America, being found from Alaska south to Mexico and as far east as the Rocky Mountains and Colorado (Linsley 1962). The adults are among the largest longhorned beetles in North America (Linsley 1962). The beetle has a long life cycle (3–5 years or more), spending most of its life underground as a root-feeding larva. The fully developed larvae pupate in spring, and the adults appear in a relatively synchronized emergence from early- to mid-summer. The nonfeeding adult life stage is relatively short (a few weeks at most), and the adults must find mates and reproduce quickly before they exhaust their energy reserves and die (JDB, pers. obs.).

Prionus californicus has a broad host range, feeding on the roots of a wide variety of perennials, and is an occasional to chronic pest of oaks, apple, cherry, almond, and many other species of woody plants in natural, agricultural, and urban environments (Solomon 1984). It is a particularly serious pest in the production of hop, *Humulus lupulus* L. (Urticales: Cannabaceae), in the northwestern USA (Bishop et al. 1984). Infestations in crops are difficult to manage because the developing larvae are protected within the roots of the host crop. Furthermore, insecticides may be ineffective against the adults because they do not feed and have a heavily sclerotized cuticle. We initiated a project to identify the sex pheromone of *P. californicus* as a first step in the possible development of noninsecticidal methods of monitoring and managing this important pest.

In preliminary studies, we showed that female *P. californicus* produce a powerful sex pheromone that specifically attracts males (Cervantes et al. 2006). The pheromone appeared to be produced by structures associated with the ovipositor, and "calling" females exhibited a stereotypical behavior of rhythmic extension and contraction of the ovipositor, with the eversion of a small sac-like projection from the dorsal surface of the ovipositor (Barbour et al. 2006). Here, we report the identification, synthesis, and preliminary field testing of a sex pheromone of *P. californicus*. This is the first female-produced sex attractant pheromone to be identified from this large beetle family, with more than 30,000 described species (Lawrence 1982).

Methods and Materials

Insects Insects were reared individually with pieces of hop root in 19-l buckets buried in the ground at the Southwest Idaho Research and Extension Center, as previously described (Cervantes et al. 2006). Each bucket was covered with a screened lid, and adult beetles were collected as they emerged. Adult beetles were shipped within 24 h after emergence by overnight courier to UC Riverside (USDA-APHIS permit 71758), where they were held at ~4°C in individual vials with a piece of moistened paper towel until used in experiments. Insects were used within 7 days after arrival at UC Riverside and, therefore, were 2–9 days old when used in experiments. Female *P. californicus* are receptive to mating within 24 h after emergence (Barbour et al. 2007).

Collection and Analysis of Pheromone Dissection and examination revealed that the eversible sac-like projection of females was contained in the ovipositor sheath, so we sampled directly from this structure. Thus, a live virgin female was held firmly while the ovipositor was gently pulled out to its full extension and clamped with a hemostat. The tip of a solid phase microextraction device fiber (polydimethylsiloxane coated fiber, 100-µm thick film, Supelco, Inc., Bellefonte PA, USA) was then inserted into the opening from which the sac-like structure was everted and moved around to adsorb constituents by contact for ~1 min. One SPME fiber was used to sequentially sample from as many as 17 females to provide sufficient adsorbed material for analysis. SPME fibers were cleaned immediately before use by thermal desorbtion at 250°C for 10 min in the injector port of a gas chromatograph. Synthetic standards (see below) were adsorbed on SPME fibers by exposing the fibers to the headspace odors from a filter paper treated with a test compound and held in a vial sealed with aluminum foil.

The loaded SPME fibers were wiped gently with a paper tissue to remove any adhering materials before analysis. Loaded fibers initially were analyzed by coupled gas chromatography-electroantennogram detection (GC-EAD), using two Hewlett-Packard 5890 series II gas chromatographs (HP, Avondale PA, USA). The first GC was fitted with a DB-5 column (30 m×0.25 mm ID, 0.25 µm film, programmed from 100°C/1 min, 10°/min to 275°C for 15 min; J&W Scientific, Folsom, CA, USA), and the second with a DB-WAX column (30 m×0.25 mm ID, 0.25 µm film, 100°C/1 min, 10°/min to 240°). Loaded SPME fibers were desorbed in the injection port for 1 min prior to starting the temperature program. Helium was used as the carrier and makeup gas, and all injections were made in splitless mode. The column effluent was split equally with a glass X-shaped union (Restek, Bellefonte PA, USA) fitted with 0.32 mm ID sections of uncoated and deactivated GC column, with one section going to the flame ionization detector (FID), another to the electroantennogram detector (EAD), and the final section providing helium makeup gas (3 ml/min). The EAD branch passed through a heated conduit (250°C) and into the side of a 15 mm ID glass tube swept with humidified medical air (770 ml/min), with the air flow directed over the insect antennal preparation. Signals were recorded on either matched HP 3394 integrators or with an SRI model 202 PeakSimple chromatography data system running on a personal computer with PeakSimple v. 2.83 software (SRI Instruments, Torrance, CA, USA).

Males used in GC-EAD analyses were not chilled or anesthetized prior to use. The five terminal flagellomeres (out of total number of 12) of one antenna were cut off, and ~1 mm of the tip was removed with a razor blade. The section of antenna then was mounted between two glass capillary electrodes filled with Locke's saline (7.5 g NaCl, 0.21 g $CaCl_2$, 0.35 g KCl, 0.2 g $NaHCO_3$ per liter distilled water), with electrical contact to the amplifier being provided with 0.2-mm gold wires. The antennal preparation was centered in the effluent air stream from the GC, and antennal signals were amplified and filtered with a custom-built amplifier and recorded in parallel with the FID signals, as described above.

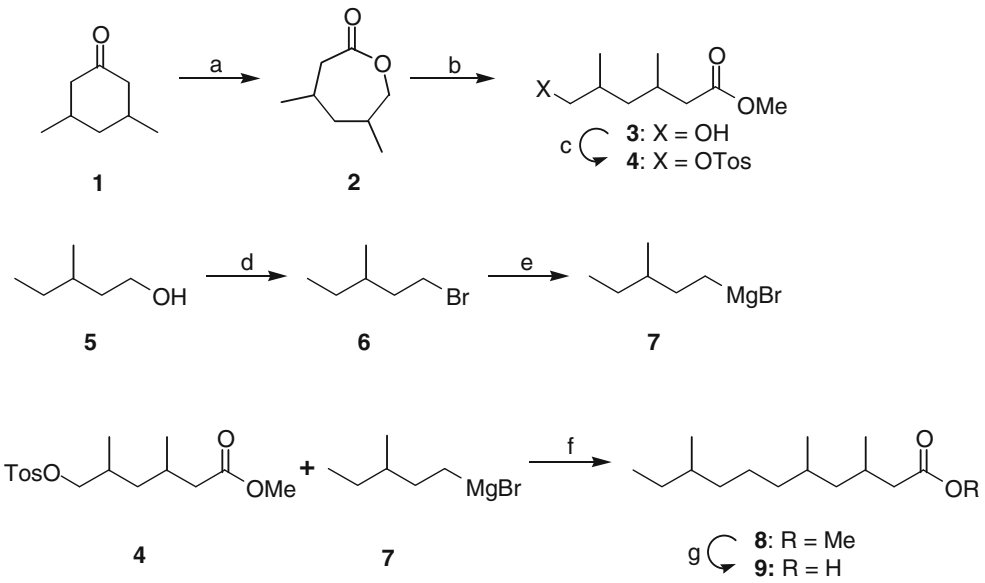
Loaded SPME fibers also were analyzed by GC-MS, using an HP 6890 GC interfaced to an HP 5973 mass selective detector (MSD), or an Agilent 6890N GC interfaced to a 5975C MSD, both fitted with HP5-MS columns (30 m× 0.25 mm ID, temperature program 40°C/0 min, 10°C/min to 280°C, hold 15 min). SPME fibers were desorbed for 30 s in splitless mode before beginning temperature programming. Kovats indices (Kovats 1965) of analytes were calculated with reference to straight-chain hydrocarbons.

Synthesis of the Pheromone and Analogs Tetrahydrofuran (THF) was distilled from sodium/benzophenone ketyl under argon. ^{1}H- and ^{13}C-NMR spectra were recorded with a Varian INOVA-400 (400 and 100.5 MHz, respectively) spectrometer, as $CDCl_3$ solutions. Chemical shifts are expressed in ppm relative to $CDCl_3$ (7.27 and 77.23 ppm for ^{1}H and ^{13}C NMR, respectively). Worked-up solutions were dried over anhydrous Na_2SO_4, and concentrated by rotary evaporation under reduced pressure. Crude products were purified by flash or vacuum flash chromatography on silica gel (230–400 mesh). Mass spectra of synthetic intermediates were obtained with a Hewlett-Packard 5890 GC interfaced to an HP 5970 mass selective detector, in EI mode (70 eV) with helium carrier gas. The GC was equipped with an HP5-MS column (25 m×0.20 mm ID ×0.33-µm film). Reactions with air- or water-sensitive reagents were carried out in dried glassware under argon atmosphere.

Syntheses of Trimethylundecanoic and 3,5-Dimethyldodecanoic Acids: Synthesis of 3,5,9-Trimethylundecanoic Acid

4,6-Dimethyl-2-Oxepanone **(2)** A solution of 3,5-dimethylcyclohexanone **1** (6.341 g, 50 mmol), *m*-chloroperbenzoic acid (approximately ~67% by weight, 12.93 g, ~50 mmol), and trifluoroacetic acid (0.384 g, 5 mmol) in methylene chloride (140 ml) was stirred in an ice bath for 1 h, then warmed to room temperature and stirred until the reaction was complete (~24 h). The mixture was then held at ~4°C overnight to precipitate most of the *m*-chlorobenzoic acid and then filtered with suction, rinsing with hexanes. The filtrate was stirred with dilute sodium bisulfite solution (75 ml) for 4 h to destroy any remaining *m*-chloroperbenzoic acid. After separation of the layers, the organic layer was washed twice with saturated sodium bicarbonate solution and brine then dried and concentrated, yielding 6.11 g (86%) of 4,6-dimethyl-2-oxepanone **2**, 96% pure by GC. This was used in the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.92 (d, J= 6.9 Hz, 3H), 0.98–1.10 (m, 1H), 1.04 (d, J=6.6 Hz, 3H), 1.25 (t, J=6 Hz, 1H), 2.0 (m, 3H), 2.49 (m, 2H), 4.05 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 19.1, 24.3, 30.0, 34.2, 42.3, 47.0, 74.4, 175.1. MS: *m*/*z* (%): 170 (2), 142 (3), 112 (55), 97 (29), 83 (9), 69 (100), 56 (45), 55 (55), 43 (28), 42 (75), 41 (89; Scheme 1).

*Methyl 6-Hydroxy-3,5-Dimethylhexanoate (**3**)* Sodium metal (0.210 g, 9 mmol) was added to dry MeOH (40 ml) in a dry three-neck flask under argon at 0°C, and the mixture was stirred until the Na had completely dissolved. 4,6-Dimethyl-2-oxepanone **2** (6.11 g, 43 mmol) was added dropwise, and the reaction was stirred at room temperature for 2.5 h, then quenched with 1 M HCl (100 ml) and extracted three times with ether. The combined ether extracts were washed sequentially with water, saturated aqueous sodium bicarbonate, and brine, then dried and concentrated. The crude methyl 6-hydroxy-3,5-dimethylhexanoate **3** (5.47 g, 87% pure by GC, 73% crude yield) was used in the next step



Scheme 1 Synthesis of 3,5,9-trimethylundecanoic acid. *a* *m*-chloroperbenzoic acid, TFA, CH_2Cl_2; *b* NaOMe, MeOH; *c* TosCl, pyridine, DMAP; *d* PBr_3, ether; *e* Mg turnings, THF; *f* Li_2CuCl_4, tosylate **4**, THF; *g* KOH, MeOH/H_2O

without further purification. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.92 (d, J=6.8 Hz, 3H), 0.95 (d, J=6.5 Hz, 3H), 1.01 (m, 2H), 1.32–1.42 (m, 1H), 1.63–1.73 (m, 1H), 1.75 (br. s, 1H), 2.0–2.08 (m, 1H), 2.11 (dd, J=14.9, 7.5, 1H), 2.30 (dd, J=14.9, 6.0, 1H), 3.48 (d, J=5.6 Hz, 2H), 3.66 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.6, 20.7, 27.8, 33.2, 40.7, 41.4, 51.7, 67.9, 174.1. MS *m/z* (%): 161 (5), 115 (6), 101 (9), 97 (5), 83 (17), 74 (100), 69 (12), 59 (22), 55 (30), 43 (29), 42 (13), 41 (39).

*Methyl 6-[[(4-methylphenyl)sulfonyl]oxy]-3,5 dimethylhexanoate (**4**)* *p*-Toluenesulphonyl chloride (10.3 g, 53 mmol) was added to a solution of 6-hydroxy-3,5-dimethylhexanoate **3** (5.47 g, 31 mmol) and dimethylaminopyridine (0.425 g, 3.4 mmol) in 40 ml pyridine at room temperature, and the mixture was stirred 12 h. The mixture was then poured into excess 1 M HCl and extracted with ether. The ether layer was washed with saturated aqueous sodium bicarbonate and brine. The sodium bicarbonate and brine layers were back extracted with ether, and the combined organic layers were dried and concentrated. The crude residue was purified by vacuum flash chromatography (95:5 hexanes:EtOAc, then 100% EtOAc) to yield 6.01 g (58.2%) of tosylate **4** of 74% purity by gas chromatography, along with 13% of recovered 4,6-dimethyl-2-oxepanone **2**. A smaller sample was repurified to obtain spectral data, yielding tosylate **4** as a 93:7 mixture of diastereomers (total, 96% chemically pure by GC). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.91 (d, J~5.6 Hz, 3H), 0.92 (d, J~6.8 Hz, 3H), 0.85–1.04 (m, 1H), 1.31 (m, 1H), 1.80–2.02 (m, 2H), 2.04 (dd, J=14.7, 8.3 Hz, 1H), 2.23 (dd, J=14.7, 5.2 Hz, 1H), 2.46 (s, 3H), 3.67 (s, 3H), 3.79 (dd, J=9.5, 6.5 Hz, 1 H), 3.90 (dd, J=9.5, 5.3 Hz, 1H), 7.36 (d, J=8.3 Hz, 2H), 7.80 (d, J=8.3 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.3, 20.5, 21.8, 27.8, 30.6, 37.8, 37.8, 40.2, 41.3, 51.7, 75.0, 128.1, 130.0, 133.3, 144.9, 173.4. MS: *m/z* (%): 328 (3), 297 (8), 254 (1), 232 (9), 173 (9), 155 (35), 141 (26), 125 (13), 114 (17), 111 (12), 101 (16), 96 (25), 91 (80), 83 (100), 74 (19), 69 (38), 55 (60), 41 (55).

*1-Bromo-3-methylpentane (**6**)* PBr_3 (4.84 g, 17.8 mmol) was added dropwise to a solution of 3-methylpentanol **5** (5.02 g, 48.8 mmol) in dry ether (65 ml) at 0°C, shielding the reaction from light. The reaction was allowed to warm to room temperature and stirred overnight. The reaction was then poured into ice water and extracted with ether. The ether layer was washed sequentially with saturated aqueous sodium bicarbonate and brine, then dried and concentrated by fractional distillation of the ether with a short Vigreux column. The resulting oil was Kugelrohr distilled (oven temp. ~70°C, 3–4 Torr) to yield 3.904 g (48.4%) of bromide **6** as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.90 (t, J=7.4 Hz, 3H), 0.90 (d, J=6.5 Hz, 3H), 1.18 (m, 1H), 1.36 (m, 1H), 1.5–1.75 (m, 2H), 1.88 (m, 1H), 3.43 (m, 2H). MS *m/z* (%): 166 (2), 164 (2), 137 (1), 135 (2), 109 (3), 107 (5), 95 (2), 93 (2), 85 (53), 69 (25), 57 (90), 41 (100).

*Methyl 3,5,9-trimethylundecanoate (**8**)* Freshly ground magnesium turnings (1.149 g, 46 mmol) were added to a dry three-neck flask under argon, and a ~4-ml aliquot of a solution of 1-bromo-3-methylpentane **6** (3.223 g, 23.6 mmol) in 20 ml dry THF was added. The mixture was stirred until the Grignard reaction started, followed by addition of the remainder of the solution by syringe pump over 1 h. After the addition was complete, the mixture was stirred for an additional 1 h then allowed to stand so that fine solids could settle out.

Dry THF (16 ml), lithium tetrachlorocuprate solution (7 ml, 0.1 M in THF, 0.7 mmol), and tosylate **4** (5.047 g, 15.3 mmol) were added to a dry three-neck flask under argon, and the mixture was chilled in an ice bath. The freshly prepared solution of 3-methylpentylmagnesium bromide **7** was added dropwise, maintaining the temperature at ~0°C. After the addition was complete, the reaction was stirred 1 h at 0°C, and then allowed to warm slowly to room temperature overnight. The reaction was then quenched with 1 M HCl, and the mixture was extracted with ether. The ether layer was washed sequentially with saturated aqueous sodium bicarbonate and brine, then dried and concentrated. The residue was purified by column chromatography (95:5 hexanes/ethyl acetate), yielding 0.45 g of the desired product **8** (12.2%) and 3.36 g (10.2 mmol, 66%) of unreacted tosylate **4.** ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.86 (m, 9 H), 0.93 (d, J=6.2 Hz, 3H), 0.95–1.17 (m, 4H), 1.18–1.38 (m, 7H), 1.46 (m, 1H), 2.05 (m, 2H), 2.31 (m, 1H), 3.67 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 11.59, 19.37, 19.45, 20.24, 20.29, 20.58, 24.44, 28.08, 29.63, 29.78, 30.22, 30.26, 34.60, 37.05, 37.12, 37.21, 41.75, 44.85, 44.91, 51.52, 174.03. MS: *m/z* (%): 185 (12), 143 (10), 131 (2), 115 (7), 101 (88), 83 (30), 74 (100), 59 (25), 55 (51), 41 (72).

*3,5,9-Trimethylundecanoic Acid (**9**)* A mixture of methanol (31 ml), water (3.4 ml), KOH (0.94 g, 17 mmol), and methyl 3,5,9-trimethylundecanoate **8** (0.201 g, 0.82 mmol) was refluxed 1 h, then cooled to room temperature. The mixture was acidified with 1 M HCl, then extracted three times with ether. The combined ether layers were washed with brine, then dried and concentrated, to yield 3,5,9-trimethylundecanoic acid **9** as a mixture of stereoisomers (0.169 g, 88.9%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.86 (m, 9H), 0.97 (two overlapped d, 3H), 0.98–1.52 (m, 10H), 2.08 (m, 2H), 2.36 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 11.60, 19.38, 20.22, 20.5, 24.42, 27.91, 29.64, 29.78, 34.60, 37.12, 41.50, 44.75, 44.82, 179.01. MS: *m/z* (%): 199 (5), 129 (100), 111 (33), 97 (22), 89 (22), 83 (10), 71 (87), 43 (37).

Synthesis of 3,5,7-Trimethylundecanoic Acid (13)

Methyl 6-iodo-3,5-dimethylhexanoate (10) A solution of methyl 6-hydroxy-3,5-dimethylhexanoate **3** (2.3 g, 13.2 mmol) and triethylamine (3.70 ml, 26.4 mmol) in ether (66 ml) was cooled to −5°C in an ice-salt bath, and methanesulphonyl chloride (1.24 ml, 15.8 mmol) was added dropwise. The reaction was stirred for 30 min, then quenched with dilute sodium bicarbonate. The organic layer was separated and washed sequentially with 1 M HCl, water, and brine, then dried and concentrated. The crude mesylate was taken up in acetone (66 ml), and NaI (6.92 g, 46 mmol) was added. The mixture was stirred 8 days at room temperature then concentrated by rotary evaporation. The residue then was partitioned between water and ether. The ether layer was washed with brine, dried, and concentrated. The residue was purified by flash chromatography (hexane/EtOAc 95:5) yielding 3.07 g (81%) of iodide **10**. ^{1}H NMR (400 MHz, CDCl3): δ 0.96 (d, 2H, J=6.5 Hz), 0.99 (d, 2H, J=6.5 Hz), 1.1 (m, 1H), 1.35 (m, 1H), 1.55 (m, 1H), 2.04 (m, 1H), 2.16 (dd, 1H, J=14.6, 8.0 Hz), 2.30 (dd, 2H, J=14.6, 5.6 Hz), 3.15 (dd, 1H, J=11.7, 5.9 Hz), 3.25 (dd, 1H, J=11.7, 4.2 Hz), 3.68 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.6, 20.2, 21.4, 28.0, 32.1, 41.7, 43.7, 51.7, 173.5. MS *m*/*z* (%): 284 (trace), 253 (6), 235 (2), 183 (2), 157 (40), 141 (3), 125 (32), 97 (36), 83 (100), 73 (17), 69 (21), 55 (92), 41 (87).

*3,5,7-Trimethylundecanoic acid (***13***)* A dry three-neck flask was loaded with freshly ground magnesium turnings (0.86 g, 35.2 mmol) and THF (17 ml) under argon. 2-Bromohexane (2.80 g, 17 mmol; Alfa Aesar) was purified immediately before use by vacuum flash chromatography on silica gel, eluting with pentane. After concentration, the bromide was added dropwise over 1 h to the slurry of Mg turnings, and the mixture was stirred a further 3 h after the addition was complete. The resulting 2-hexylmagnesium bromide solution **11** was then added dropwise to an ice-bath-cooled solution of lithium tetrachlorocuprate (1.8 ml, 0.18 mmol), THF (5 ml), and methyl 6-iodo-3,5-dimethylhexanoate **10** (1.0 g, 3.51 mmol). The reaction was held at ~0°C for 2 h then allowed to warm slowly to room temperature overnight. The mixture was quenched with 1 M HCl and extracted with ether. The ether layer was washed sequentially with saturated sodium bicarbonate and brine, dried, and concentrated. The resulting crude methyl 3,5,7-trimethylundecanoate **12** was refluxed 1 h in a mixture of methanol (100 ml), water (10 ml), and KOH (4.02 g, 71.6 mmol). After cooling, the mixture was diluted with water and any unreacted methyl ester and Wurtz coupling dimerization products from the Grignard reagent were removed by extracting the mixture twice with ether. The remaining aqueous layer was then acidified with HCl and extracted three times with ether. The combined ether extracts were washed with brine, dried, and concentrated, yielding 240 mg of crude acid **13**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.78–0.92 (m, 9H), 0.93–0.99 (m, 3H), 0.99–1.14 (m, ~4H), 1.14–1.37 (m, ~7H), 1.37–1.51 (m, 1H), 1.51–1.62 (m, 1H), 2.07 (m, 2H), 2.37 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.40, 19.57, 20.14, 20.31, 20.54, 20.59, 20.73, 20.78, 23.23, 23.74, 27.52, 27.62, 27.77, 27.87, 29.33, 29.54, 30.06, 30.15, 36.41, 37.99, 38.01, 41.34, 41.69, 44.48, 44.88, 45.25, 45.60, 179.40. MS *m*/*z* (%): 228 (1), 210 (1), 192 (5), 171 (7), 169 (7), 153 (12), 143 (6), 139 (4), 129 (33), 111 (32), 101 (23), 87 (100), 69 (49), 57 (45), 43 (62).

Synthesis of 3,5-Dimethyldodecanoic Acid (**18**)

*1-Bromo-2-methylnonane (***15***)* Mesyl choride (9.58 ml, 123 mmol) was added dropwise to a chilled solution (~10°C in an ice-salt bath) of 2-methylnonanol **14** (18.72 g, 112 mmol) in methylene chloride (280 ml) and triethylamine (23.2 ml, 168 mmol). The resulting mixture was stirred 1 h at ~−10°C then filtered with suction. The filtrate was washed sequentially with 1 M HCl, saturated $NaHCO_3$, and brine, then dried and concentrated. The crude mesylate was taken up in acetone (280 ml), and LiBr (29.11 g, 336 mmol) was added in three portions (exothermic!), allowing the mixture to cool between additions of each portion. The mixture was stirred 3 h at room temperature then heated to 60°C overnight. After cooling to room temperature, the mixture was filtered with suction, and the filtrate was concentrated. The crude reaction mixture was diluted with pentane and washed with water and brine. After concentration, the residue was purified by vacuum flash chromatography on silica gel, eluting with pentane. The purified 1-bromo-2-methylnonane **15** then was Kugelrohr distilled (oven temp ~65°C: 1.6 Torr), yielding 21.68 g of colorless oil (85%), 91% pure by GC. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.89 (t, J=6.78 Hz, 3H), 1.01 (d, J=6.62 Hz, 3H), 1.28 (m, 10H), 1.45 (m, 1H), 1.80 (m, 1H), 3.33 (dd, 1H, J=9.75, 6.24 Hz), 3.40 (dd, 1H, J=9.75, 4.94 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.29, 18.99, 22.85, 27.08, 29.44, 29.86, 32.04, 35.07, 35.41, 41.83. MS *m*/*z* (%): 113 (trace), 98 (16), 85 (17), 71 (31), 57 (86), 41 (100).

*3,5-Dimethyldodecanoic Acid (***18***)* 1-Bromo-2-methylnonane **15** (11.57 g, 52.3 mmol) was added dropwise to a slurry of Mg turnings (3.64 g, 150 mmol) in dry THF (96 ml) under argon over ~2 h. The resulting solution of Grignard reagent **16** was then added by syringe to a slurry of CuI (0.678 g, 3.56 mmol) and dry THF (10 ml) in a dry three-neck flask cooled to 0°C under argon. Then, β-butyrolactone **17** (5.38 g, 62.5 mmol) in dry THF (44.6 ml) was added

dropwise over 60 min. The mixture was stirred at 0°C for 30 min and then warmed to room temperature overnight. The reaction was quenched with 1 M HCl and extracted with ether three times. The combined ether extracts were washed twice with 1 M NaOH. The resulting combined aqueous layers then were acidified with 3 M HCl, and extracted twice with ether. The combined ether layers were washed with brine, dried, and concentrated to give 6.7 g of crude acid **18** (47%). Attempted Kugelrohr distillation of a portion of the crude acid resulted in decomposition, and so the remainder (6.7 g) was purified by flash chromatography (hexanes/EtOAc 3:1, with 1% acetic acid), giving 6.1 g of 3,5-dimethyldodecanoic acid **18** of 96% purity by GC. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.86 (t, 3H, J=6.6 Hz), 0.88 and 0.90 (dd, 3H, J=6.7 Hz), 0.95 and 0.97 (dd, 3H, J=6.4 Hz), 1.00–1.20 (m, 2H), 1.27 (m, 12H), 1.47 (m, 1H), 2.10 (m, 1H), 2.3 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.3, 19.5, 19.6, 20.2, 20.5, 22.9, 27.0, 27.2, 27.9, 27.9, 29.6, 30.1, 30.2, 32.1, 36.8, 37.9, 41.5, 42.4, 44.5, 44.8, 179.1, 179.3 (MS: see Fig. 2C).

Field Bioassay of Synthetic Pheromone A field bioassay of the synthetic 3,5-dimethyldodecanoic acid was conducted at Placerita Canyon Natural Area (Los Angeles Co., CA), an area where adult *P. californicus* frequently are collected in light traps during summer months (I. P. Swift, pers. obs.). The bioassay was deployed from 12 to 17 June 2008 (clear skies, no precipitation, maximum air temperatures 21–36°C). Traps consisted of 19-l polypropylene buckets (38 cm tall× 30 cm diam) fitted with aluminum funnels (model 2815B, BioQuip, Rancho Dominguez, CA, USA) that were buried with the top of the funnel flush with the soil surface. Lures consisted of clear low density polyethylene press-seal bags (Bagette model 14770, 5.1×7.6 cm, 0.05 mm wall thickness, Cousin Corp., Largo, FL, USA) that were loaded with 100 mg of synthetic 3,5-dimethyldodecanoic acid (as the mixture of four stereoisomers) in 900 µl ethanol. Control lures consisted of bags loaded with 1 ml of pure ethanol. Lures were hung inside trap funnels with wire. Traps were set up in a westerly transect in an area wooded with mature *Quercus agrifolia* Neé (position of first trap N 34°22′42″ W 118°28′53″, 462 m elevation). There were two sets of traps in the transect, each including two traps baited with pheromone and two control traps, with traps spaced ~8 m apart, and with the sets separated by 60 m. Traps were checked for beetles daily at ~8:00 AM.

Differences between treatments in numbers of beetles captured were tested with the nonparametric Friedman's test (blocked by set and day, PROC FREQ with CMH option; SAS Institute 2001) because a mean of zero for controls violated assumptions of analysis of variance (Sokal and Rohlf 1995). We used a mark-recapture technique to estimate the local abundance of male *P. californicus*. All beetles that were removed from traps on 13–16 June 2008 were marked with a spot of blue fingernail polish on the pronotum and were released in wooded areas at least 20 m from traps. We recorded the number of marked individuals that were recaptured and used the Lincoln-Peterson index to estimate the size of the local population based on the accumulated number of marked beetles that had been released (Southwood and Henderson 2000).

Voucher specimens of *P. californicus* have been deposited at the Entomology Research Museum at the University of California, Riverside, CA.

Results

Identification of the Pheromone Gas chromatography-electroantennogram detection analyses of the compounds collected from female beetles by contact SPME showed that the antennae of male *P. californicus* responded strongly to a compound present in nanogram quantities (estimated from peak size in GC-MS total ion current chromatograms) in the extracts (Fig. 1, peak 2), with additional responses to several other components (Fig. 1, peaks 1, 3, and 4). Analyses of SPME samples by GC-MS produced a mass spectrum (Fig. 2A) that suggested that the most active compound had a probable molecular weight of 228 Da, for a possible molecular formula of $C_{14}H_{28}O_2$, which would accommodate one site of unsaturation. The distorted peak

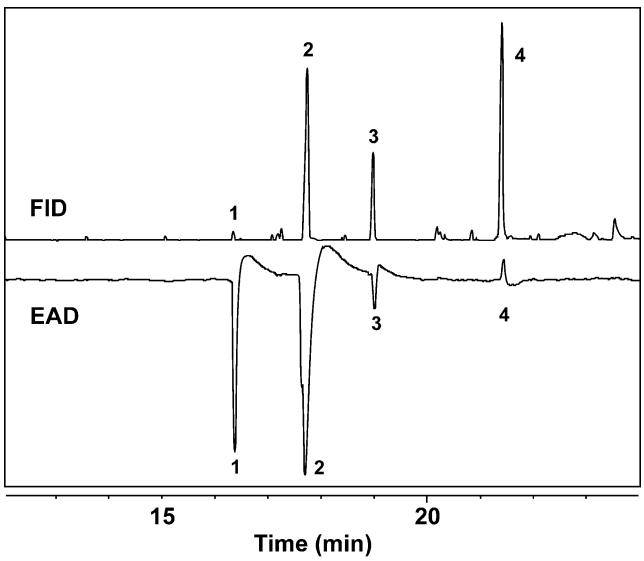


Fig. 1 Representative coupled gas chromatography-electroantennogram analysis of a contact SPME extract of the ovipositor of a female *P. californicus*. *Upper trace* is the chromatogram from a DB-5 GC column, *lower, inverted trace* is the antennal response. *Peak 1* = methyl 3,5-dimethyldodecanoate; *peak 2* = 3,5-dimethyldodecanoic acid; *peak 3* = 3,5-dimethyltridecanoic acid; *peak 4* = 3,5-dimethylpentadecanoic acid

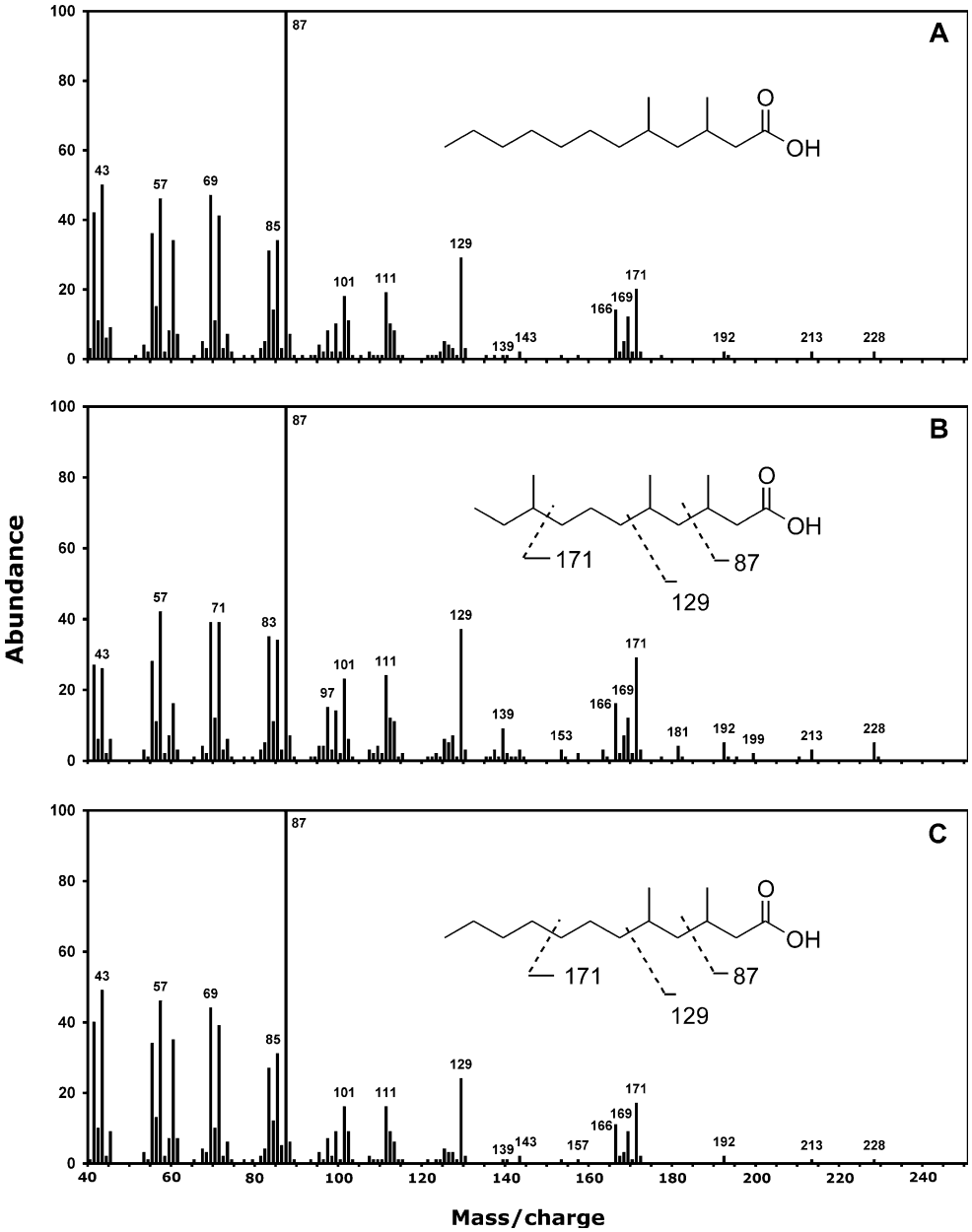


Fig. 2 Electron impact ionization mass spectra of: **A**) the compound from the SPME extract of female ovipositor that elicited the largest antennal response from males (Peak 2 in Fig. 1, 3,5-dimethyldodecanoic acid), **B**) a stereoisomer of 3,5,9-trimethylundecanoic acid, and **C**) the synthetic diastereomer of 3,5-dimethyldodecanoic acid with the same retention time as the insect-produced compound

shape suggested a polar analyte such as a diol or carboxylic acid. Further evidence of a polar analyte was obtained by comparison of the Kovats indices (KI; Van den Dool and Kratz 1963; Kovats 1965) of the compound on a nonpolar DB-5 column (1636) vs. the KI value on a polar DB-WAX column (2541). The large difference between the two columns (905 KI units) was virtually identical to the corresponding difference in KI values for a model compound, tridecanoic acid, on DB-5 (1677) and DB-WAX (2585, difference 908 KI units). The carboxyl function accounted for the single site of unsaturation, indicating that the compound could have no other double bonds or rings. Furthermore, the fact that the KI values of the insect-produced compound were actually lower than those of tridecanoic acid, even though tridecanoic acid contained one less carbon, indicated that the compound was a branched rather than a straight-chain structure.

Detailed examination of the mass spectrum (Fig. 2A) provided further information. The base peak at *m/z* 87 was tentatively assigned to a carboxylic acid with a methyl branch at the carbon ß to the carboxyl function (C_3). A second significant fragment at *m/z* 129 suggested a second methyl branch at C_5. The ion at *m/z* 171 suggested a third methyl branch at either C_7 or at C_9 if cleavage had occurred closer to the end of the chain. Other useful fragments included a relatively strong *m/z* 60 ion, from loss of acetic acid, and even-mass ions at *m/z* 192 (loss of $2\times H_2O$) and 166 (loss of $C_2H_6O_2$).

The additional component that elicited the first antennal response (Fig. 1, peak 1) was tentatively identified as the methyl ester of the major pheromone component and provided further support for some of the structural elements of the parent compound. Its mass spectrum was characterized by a base peak at *m/z* 74 from McLafferty rearrangement of a methyl ester with two hydrogen on the carbon α to the carbonyl group, a large fragment at *m/z* 101 (65%) from cleavage of a methyl ester with a methyl branch on C_3, a weak molecular ion at *m/z* 242 (2%), and two significant fragments at *m/z* 143 (10%) and 185 (9%), analogous to the fragments at *m/z* 129 and 171 in the spectrum of the parent acid, after accounting for the additional 14 mass units from the methyl ester.

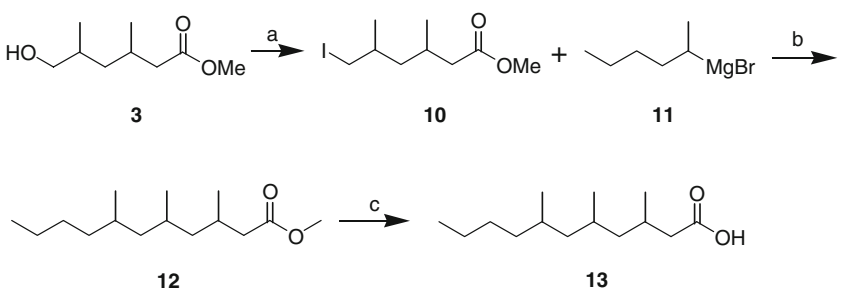
Based on the above data, the 3,5,9-trimethylundecanoic acid structure was deemed the most likely structure, and this compound was synthesized as a mixture of all eight stereoisomers, which appeared as three broad, overlapping peaks on GC analysis. The mass spectra of the synthetic 3,5,9-trimethylundecanoic acid isomers were similar but not quite identical to that of the insect-produced compound (Fig. 2B). One of the key discriminating factors was the difference in the ratio of the low intensity ions at *m/z* 139 and 143 between the spectra of all of the 3,5,9-trimethylundecanoic acids and the natural compound. Because of the uncertainty in measuring the Kovats indices of these broad and overlapping peaks, which also were concentration-dependent, the mixture of acids was methylated, and the KI values of the methyl esters were compared to that of the trace amount of the methyl ester of the pheromone that was present in the insect extract (Fig. 1, peak 1). The mixture of methyl ester diastereomers appeared as two peaks on the DB-5 column, and the KI values of both were significantly shorter (1558 and 1567) than that of the insect-produced compound (1600), proving that the 3,5,9-trimethylundecanoic acid structure was not correct. Furthermore, the synthetic material was virtually inactive in both laboratory and field bioassays (JDB, unpublished data).

We reasoned that another trimethyl branched alternative, i.e., 3,5,7-trimethylundecanoic acid, was even less likely to be correct because shifting the outermost methyl group inwards towards the middle of a hydrocarbon chain would further decrease the retention time. This was verified by synthesis of standards; the KI values of the four diastereomeric methyl esters (1518, 1526, 1538, 1547) were all considerably lower than that of the insect produced compound, and the mass spectra were all obviously different than that of the natural product.

A third trimethyl-branched alternative, 3,5,9-trimethylundecanoic acid, also was ruled out by synthesis of standards; the mass spectrum of the natural compound and those of the synthetic isomers were markedly different, and the Kovats indices on DB-5 (1569, 1574, 1608) of the three peaks from the methyl esters of the mixture of synthetic stereoisomers did not match that of the insect-produced compound.

Having eliminated what seemed to be the most reasonable structures based on the mass spectral data, and with the information that the Kovats indices suggested a longer chain with fewer branch points, we then considered which dimethyldodecanoic acids might fit the data. Of the significant mass spectral fragments, the assignment of the *m/z* 87 base peak as a fragment arising from cleavage of a carboxylic acid with a methyl branch at C_3 seemed robust, based on examination of database spectra of saturated carboxylic acids with a methyl group in the three position. Thus, the most likely positions for the remaining methyl group were at either C_5 (supporting the fragment at *m/z* 129) or C_8 (supporting the fragment at *m/z* 171), with biosynthetic considerations favoring the former. Nonstereoselective synthesis of 3,5-dimethyldodecanoic acid resulted in a mixture in which the *syn* and *anti*-diastereomers were separable by GC, with the later eluting peak matching the natural compound in both Kovats index and mass spectrum (Fig. 2A vs C). Further confirmation of the identification was obtained from the excellent match between the mass spectrum and Kovats index of the corresponding methyl ester with those of the insect-produced methyl ester (Fig. 1, peak 1). In total, these data confirmed that the pheromone was one of the stereoisomers of 3,5-dimethyldodecanoic acid and that the ion at *m/z* 171, despite its prominence, was not indicative of a branch point.

In addition to 3,5-dimethyldodecanoic acid and its methyl ester (Fig. 1, peaks 2 and 1, respectively), the insect extracts also contained two compounds tentatively identified as the homologs, 3,5-dimethyltridecanoic and 3,5-dimethylpentadecanoic acid (Fig. 1, peaks 3 and 4, respectively) based on the facts that their Kovats indices were approximately 100 and 300 units greater than that of the major pheromone component, and the analogies between their mass spectra and the mass spectra of 3,5-dimethyldodecanoic acid. In particular, their mass spectra were similar to that of 3,5-dimethyldodecanoic acid in the lower mass ranges, being characterized by base peaks at *m/z* 87 and significant ions at *m/z* 129, indicative of methyl branches on C_3 and C_5, and a significant *m/z* 60 ion from loss of acetic acid. However, their molecular ions were integer multiples of 14 mass units higher than that of 3,5-dimethyldodecanoic acid, and the cluster of ions at *m/z* 166, 169, and 171 corresponding to losses of 62, 59, and 57 Da from the molecular ion of 3,5-dimethyldodecanoic acid at *m/z* 228, was replaced with analogous clusters of ions at *m/z* 180, 183, and 185 Da, and 208, 211, and 213 Da, respectively. A trace amount of the corresponding 3,5-dimethyltetradecanoic acid was also tentatively identified from its base peak at *m/z* 87, a large *m/z* 129 ion, and a cluster of ions at *m/z* 194, 197, and 199. These identifications have not yet been confirmed



Scheme 2 Synthesis of 3,5,7-trimethylundecanoic acid. *a* MesCl, Et_3N, ether; NaI, acetone; *b* CuI, iodide **10**, THF; *c* KOH, $MeOH/H_2O$

by synthesis, but the presence of the diagnostic patterns of ions and the Kovats index values provide substantial support for the proposed structures. The full EI mass spectra of these and related compounds are provided in the online supplement.

Syntheses of Trimethylundecanoic and 3,5-Dimethyldodecanoic Acids A nonstereoselective synthesis of 3,5,9-dimethyldodecanoic acid was developed (Scheme 1) so that all possible isomers would be represented in the resulting mixture. Thus, 3,5-dimethylcyclohexanone was subjected to Baeyer–Williger oxidation with *m*-chloroperbenzoic acid, yielding 4,6-dimethyl-2-oxepanone **2**. The lactone was transesterified with sodium methoxide in methanol to provide methyl 6-hydroxy-3,5-dimethylhexanoate **3**, which was then converted to tosylate **4**. A second synthon was constructed by conversion of 3-methylpentanol **5** to bromide **6** by treatment of **5** with PBr_3. The Grignard reagent **7** prepared from bromide **6** was then reacted with tosylate **4** with Li_2CuCl_4 catalysis, to produce methyl 3,5,9-trimethylundecanoate **8**. Basic hydrolysis of the ester in aqueous ethanol gave the free acid **9**.

The second possibility, 3,5,7-trimethylundecanoic acid, was constructed using similar methodology (Scheme 2). Thus, methyl 6-hydroxy-3,5-dimethylhexanoate **3** from the above synthesis was converted to the corresponding iodide **10** via the mesylate. Reaction of the iodide with 2-hexylmagnesium bromide, with CuI catalysis, produced methyl 3,5,7-trimethylundecanoate **12**. Basic hydrolysis of the ester gave the free acid **13**.

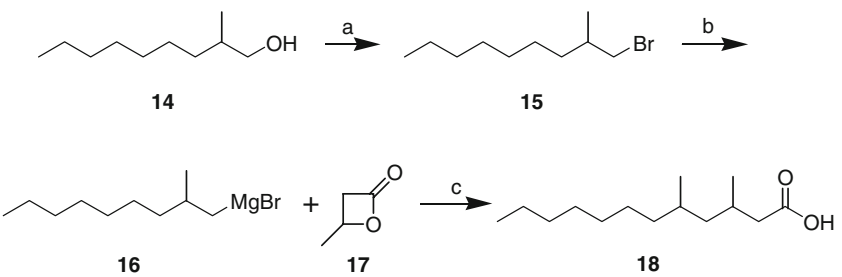
3,5-Dimethyldodecanoic acid was synthesized nonstereoselectively (Scheme 3) by ring opening of ß-butyrolactone with 2-methylnonylmagnesium bromide, with copper catalysis (Sato et al. 1980), yielding 3,5-dimethyldodecanoic acid **19** in a single step.

Field Bioassay Synthetic 3,5-dimethyldodecanoic acid as the mixture of all four stereoisomers attracted male *P. californicus* in a field bioassay. An average (± SE) of 3.5± 0.34 male beetles per trap per day were captured in traps baited with the synthetic pheromones (total over 5 days, 72 beetles), whereas no beetles were captured in control traps (means significantly different; Friedman's $Q_{1,39}=27.6$, $P<0.001$). No females were captured. In the mark-recapture portion of the assay, we recaptured only eight males that had been marked with paint and released, and Lincoln–Peterson estimates of local population size averaged (± SD) 280±230 male *P. californicus*.

Discussion

Whole body extracts of female *P. californicus* have been shown to be highly attractive to males (Barbour et al. 2006), but they contain a plethora of cuticular hydrocarbons and other contaminants that obscure possible pheromone components. Similarly, attempts to collect sufficient pheromone for analysis by using dynamic headspace collection, trapping odors released by unmated female beetles on activated charcoal, were unsuccessful; after elution with both polar and nonpolar solvents, the resulting extracts were minimally attractive to male beetles. Analyses of the compounds absorbed from the inside of the ovipositor, however, showed that the main pheromone component had been collected in detectable amounts by this method, along with several homologs.

The identification of the pheromone was confounded by the small amounts of pheromone that could be recovered in insect extracts, and by the fact that the contact SPME method of extracting and analyzing the pheromone provided



Scheme 3 Synthesis of 3,5-dimethyldodecanoic acid. *a* MesCl, Et_3N, CH_2Cl_2; LiBr, acetone; *b* Mg turnings, THF; *c* CuI, ß-butyrolactone **17**

only mass spectral and retention time data. The combination of both types of data proved crucial in finally identifying the correct structure. The remarkable similarity between the mass spectra of the synthetic 3,5,9-trimethylundecanoic acid and the pheromone at first led us to believe that this structure was correct, but retention time comparisons on both the polar and nonpolar columns, with both the free acids and the corresponding methyl esters, proved that this was not possible. Detailed examination of the mass spectra showed that there were indeed small but reproducible differences between the spectra. Nevertheless, the presence of the misleading *m/z* 171 ion, and the corresponding ions in the homologs, is noteworthy. In fact, it had been previously noted that even straight-chain carboxylic acids display periodic enlarged fragment ions in the middle of the aliphatic chain, with no obvious source of stabilization to account for the fragmentation (McLafferty and Turecek 1993, p. 218).

Only male beetles were caught in traps baited with the synthetic 3,5-dimethyldodecanoic acid, thus verifying that this compound is a sex pheromone. The substantial number of beetles captured also suggests two other points. First, the fact that the beetles were attracted to the mixture of all four stereoisomers of 3,5-dimethyldodecanoic acid suggests that none of the "unnatural" stereoisomers are likely to be strongly antagonistic. The lack of antagonism will be a tremendous benefit for the commercialization of the pheromone for monitoring and/or control of the beetle, because as described above, 3,5-dimethyldodecanoic acid can be readily prepared in multigram quantities in a single step from two simple precursors. The relatively strong attraction obtained with 3,5-dimethyldodecanoic acid also suggests that the other compounds in the extracts from females are not crucial components of the pheromone, and that racemic 3,5-dimethyldodecanoic acid as a "single" component is a satisfactory trap bait.

To our knowledge, the work described here represents the first identification of a pheromone for a species in the longhorned beetle subfamily Prioninae. However, there is considerable circumstantial evidence that female-produced sex attractant pheromones are widespread within the Prioninae (e.g., Rotrou 1936; Edwards 1961; Benham and Farrar 1976; Gwynne and Hostetler 1978), so we anticipate that pheromones of other species, particularly congeners of *P. californicus*, will soon be reported, now that the basic methods and a "lead" compound have been characterized. It should also be noted that the pheromone of *P. californicus* is entirely different in structure from the male-produced pheromones of species in other subfamilies of the Cerambycidae (e.g., Hanks et al. 2007; Ray et al. 2009), and female-produced pheromones of two species that are apparently closely related to the Cerambycidae: *Migdolus fryanus* Westwood and *Vesperus xatarti* Dufour (Leal et al. 1994; Boyer et al. 1997; taxonomy according to Napp 1994; Bense 1995; Dong and Yang 2003).

There is still considerable work to be done to complete the identification of the pheromone of *P. californicus*, and to use it most effectively for insect management. First, further details of the pheromone itself (e.g., which stereoisomer[s] are produced by the beetle, and the possible role of the other components in extracts) need to be worked out. Second, operational details of using the pheromone efficiently for sampling and control of this insect require optimization. Unlike some other groups of insects for which numerous pheromones are known and for which traps and pheromone release devices have been well studied, relatively little has been done with pheromone trapping of cerambycids. For example, the carboxylic acid chemistry of the *P. californicus* pheromone is unusual among insect pheromones, and release devices such as the rubber septa that are commonly used for many insect pheromones do not work well with this compound (unpub. data). Furthermore, our field observations suggest that the beetles may walk or fly towards pheromone sources, so traps should be designed to be accessible to both walking and flying insects. These and related questions will be addressed in ongoing research, so that this novel pheromone can be fully developed for management of *P. californicus* in crops where it is a chronic problem.

The online supplement to this manuscript includes the full EI mass spectra of EI mass spectrum of 3,5,7-trimethylundecanoic acid, 3,5,9-trimethylundecanoic acid, 3,5-dimethyldodecanoic acid, 3,5-dimethyltridecanoic acid, 3,5-dimethylpentadecanoic acid, methyl 3,5,7-trimethylundecanoate, methyl 3,5,9-trimethylundecanoate, and methyl 3,5-dimethyldodecanoate.

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Identification of Chemicals Emitted by Calling Males of the Sapote Fruit Fly, *Anastrepha serpentina*

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Abstract Emissions from sexually active *Anastrepha serpentina* males were collected by solid-phase microextraction. Calling behavior of wild-type males showed no clear peak during the day, except that it was evident less frequently immediately after daybreak and just before dark. Calling by laboratory males was highest between 8 and 11 h after onset of the photophase, and mating by wild flies occurred mostly between 6 and 10 h after onset of the photophase. Two major components of male emissions were identified as 2,5-dimethylpyrazine (DMP) and 3,6-dihydro-2,5-dimethylpyrazine (DHDMP). DHDMP was synthesized, and the identity of the natural product confirmed by comparison of gas chromatographic retention times and mass spectrum. Emissions of DMP and DHMP were greatest during peak calling behavior, with males emitting up to 1.8 and 3.3 μg/h of DMP and DHDMP, respectively. A minor component, which did not vary with time of day, was identified as 2,3,5-trimethylpyrazine. To our knowledge, this is the first report of 3,6-dihydro-2,5-dimethylpyrazine in nature.

Keywords Solid phase microextraction (SPME) · Sex pheromone · Pyrazine · Dihydropyrazine · Diptera · Tephritidae

Introduction

Anastrepha serpentina is known as the "Sapote (Zapote in Spanish) fruit fly" as it principally infests fruit within the Sapotaceae [e.g., *Calocarpum mammosum* (L.) P. Royen, *Chrysophyllum mexicanum* Brandegee ex. Stand, *Chrysophyllum cainito* L., *Bumelia sebolana* Lundell, *Manilkara zapota* (L.) P. Royen, *Pouteria campechiana* (Kunth) Baehni, *Pouteria glomerata*, *Pouteria obovata* H.B.K., *Pouteria hypoglauca* (Standley) Baehni, *Sideroxylon capiri* (A.DC.) Pittier] (Baker et al. 1944; Aluja et al. 1987, 2000b, 2003; Norrbom 2004). On occasion, it has been reported to infest citrus [e.g., *Citrus aurantium* L., *Citrus sinensis* (L.) Osbeck, both Rutaceae], mango (*Mangifera indica* L., Anacardiaceae), peaches and pears [*Prunus persica* (L.) Batsch, and *Pyrus communis* L., respectively, both Rosaceae] (Baker et al. 1944, Aluja et al. 1987, Norrbom 2004), and is thus considered a pest of quarantine significance (Sharp et al. 1989). It is distributed from southern USA to Argentina, excluding the Caribbean Islands (Hernández-Ortíz and Aluja 1993).

The sex and aggregation pheromones of *Anastrepha* fruit flies have been investigated for only a few pest species of the nearly 200 species in the genus (Heath et al. 2000). The

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first chemicals identified from male emissions in this genus were (*Z*)-3-nonenol and (*Z,Z*)-3,6-nonadienol from *Anastrepha ludens* (Esponda-Gaxiola 1977). The same alcohols and two novel diastereomeric pairs of enantiomeric bicyclic lactones, which were subsequently named anastrephin and epianastrephin, were next identified from *Anastrepha suspensa* and *A. ludens* (Nation 1983; Battiste et al. 1983; Stokes et al. 1983). Later, a novel macrolide, subsequently named suspensolide (Chuman et al. 1988), and (*E,E*)-α-farnesene were identified from both of these species, and β-ocimene and β-bisabolene were found in emissions of *A. suspensa* (Rocca et al. 1992; Heath et al. 2000). Several of these compounds and their combinations were attractive to sexually active female flies in laboratory assays (Nation 1975, Robacker and Hart 1985), but many of the chemicals have not been tested.

Work on pheromones of other *Anastrepha* has been less extensive. During the early 1990s, pheromones from additional species were investigated for the purpose of developing attractants as well as understanding phylogenetic relationships within the genus. Heath et al. (2000) collected and quantified emissions from *Anastrepha obliqua*, *Anastrepha striata*, *Anastrepha fraterculus* (Central American populations), and *A. serpentina*, but none of the chemicals were tested for pheromonal activity. Principal compounds in emissions of *A. obliqua* were (*Z*)-3-nonenol, (*E,E*)-α-farnesene, and (*Z,E*)-α-farnesene, of *A. striata*, linalool, ethyl hexanoate, and ethyl octanoate, and of *A. fraterculus*, nonenols, anastrephin, and epianastrephin. De Lima et al. (1996) reported (*Z,E*)-α-farnesene, (*E,Z*)-α-farnesene, suspensolide, and a series of pyrazines in male salivary glands of Brazilian *A. fraterculus*. Pheromonal activity was not demonstrated for any of these.

Although collections of male emissions were made for *A. serpentina*, no male-specific chemicals were reported (Heath et al. 2000). Lek formation in this species, and behavior of flies in leks under natural conditions (Aluja et al. 1989), as well as a description of the microbehaviors that together make up "calling" (Aluja et al. 2000a) have been studied. Briefly, calling includes puffing of the pleural areas of the abdomen, eversion of a presumed pheromone gland from the abdominal tip and touching the gland to a leaf surface, and rapid wing vibrations often accompanied by the male spinning on the leaf surface. In addition, Castrejon-Gomez et al. (2007) investigated other aspects of the calling behavior of this fly, reporting effects of age, time of day, and male density on calling by mass-reared and wild males. Nothing has been published about the chemical composition of the emissions of calling males.

The purpose of this work was to identify the principal male-specific chemicals in emissions of male *A. serpentina*. For this work, we collected male emissions by using solid-phase microextraction, a technique different from those used previously for this and the other species of *Anastrepha*.

Methods and Materials

Insects and Test Conditions Observations of calling behavior were conducted with feral flies collected from *Chrysolphyllum mexicanum* collected in the vicinity of Teocelo, Veracruz, Mexico (19°23′ N, 96°58′ W) and from *M. zapota* collected in the vicinity of Metapa de Domínguez, Chiapas, Mexico (14°50′ N, 92°11′ W). Fruit was transported to the laboratory in Xalapa, Veracruz and placed in plastic baskets that, in turn, were placed over plastic washbowls containing vermiculite as a pupating medium. All pupae were weighed and sorted by size. To reduce any size effect on sexual performance, we used only pupae that weighed between 10 and 20 mg.

All other work was conducted with flies from a laboratory culture that originated from mamey fruit (*Pouteria sapota*; Sapotaceae), a native host of the fly, collected in Chiapas in 2000, and reared on artificial diet for at least 25 generations. Diet ingredients include maseca, torula yeast, sucrose, vitamins, beck's salt, methyl paraben, sodium benzoate, agar, HCl, and cholesterol. Flies were segregated by sex 2–3 days after eclosion. Flies in the laboratory culture have not been observed to mate before 7 days after eclosion. Males used for collections of volatiles were put into Plexiglas cages (20.5× 20.5×20.5 cm) with screened tops. Cages were provisioned with a protein/sugar mixture and additional sugar in a separate container, as well as water. Laboratory conditions for holding and testing flies were 24±2°C, 60±20% relative humidity, and a 13-h photophase that began at 0330 h and ended at 1630 h, with exceptions specified later. Volatiles collections were conducted in the same room where adult test flies were held. The room contained inlet and outlet vents to bring new air into the room from the outdoors and expel air from the room. Complete air replacement in the room occurred eight times per hour. Overhead lighting was provided by fluorescent "cool white" lights (F40CW, General Electric).

Observations of Calling Behavior of Males in Field Cages At 8 days of age, all individuals, sorted by geographical origin (i.e., Veracruz and Chiapas) were marked with a distinctive color on the pronotum (Veracruz—white, Chiapas—green) with water-based paint (Vinci, Vinci de Mexico S.A. de C. V. Mexico; Aluja et al. 2001). Observations were carried out inside a cylindrical field cage (3 m diam.×3 m height) placed inside a climate-controlled room (25±2°C, 70% RH and 0–120 lx light intensity, simulating daily patterns) at the Instituto de Ecologia, A.C., Xalapa, Veracruz (19°30′ N and 96°55′ W, 1,300 m elevation). To simulate a patch of

host and non-host trees, sapodilla (*M. zapota*), tropical plum (*Spondias mombin* L., Anacardiaceae), orange (*C. sinensis*), and mango (*M. indica*) potted trees were placed on wooden stools and arranged in the center of the field cage as described in Aluja et al. (2001).

Over a 5-day period, ten virgin females and ten virgin males from each population (total of 40 flies) were released into the cage daily and removed at the end of the day. Males were released the night previous to testing (21:00 h) and females the day of testing (05:30 h). Starting at 06:00 h and until 20:00 h, all calling males and mating pairs were counted every 15 min. A male was considered to be calling if the pheromone gland was everted from the abdomen tip, or if he engaged in rapid wing vibrations.

Collection and Gas Chromatographic Analysis of Male Emissions Volatiles emitted by sexually mature males were collected by using solid phase microextraction (SPME) with a polydimethylsiloxane (PDMS) coated fiber (100 μm coating; Supelco, Inc., Bellefonte, PA, USA). Twenty sexually mature, virgin males (8–23 days old) were put into a 2-neck 500-ml round-bottom flask with ground glass joints, early in the morning of the day when collections were to be conducted. Collections of volatiles were also done using 20 virgin, sexually mature females (15–18 days old) three times on each of 2 days. Compressed air entered the flask through a ground glass gas inlet tube that extended into the flask to within 2 cm of the flask wall on the opposite side. Flow rate was set to 200 ml min^{-1} by using a VICI Condyne flow controller (Valco Instruments Co., Inc., Houston, TX, USA). Air exited the flask through the second neck via a ground glass hose adapter, modified by replacing the hose connector with a 4.5-mm i.d. glass tube. This arrangement allowed the air to sweep through the entire flask before exiting. The glass tubes for incoming and exiting air were loosely packed with a piece of tissue as a barricade to the flies. The flask was turned upside down so that incoming air tube emptied onto the upper wall of the flask and outgoing air exited on the bottom. Calling flies congregated on the upper wall because a large green leaf was taped onto the top on the outside of the flask so as to allow flies to sit on the "underside" of a leaf, a typical site for calling *Anastrepha* on trees (Aluja et al. 2000a). Additional light was provided above the leaf with a 75-W incandescent light bulb, creating shadow on the underside of the leaf.

Volatiles were collected by inserting the SPME sheath 2 cm into the 4.5-mm ID tube and extending the PDMS fiber for 1 h per sampling. Most samplings were done from 0800 to 1630 h at 24°C. These times corresponded to 4.5 h after onset until the end of the photophase. For some collections, the onset of the photophase was adjusted to later in the day so that emissions early in the photophase could be sampled conveniently. Volatiles were analyzed by gas chromatography (GC) after thermal desorption from the PDMS fiber by on-column injection at 220°C in a 15-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 J&W DB-1 (30 m, 0.32 mm ID, 5 μm film; Agilent Technologies, Inc., Santa Clara, CA, USA). Column oven temperature was 50°C for 5 min, then programmed at 5°C min^{-1} to 200°C. Carrier gas was helium at a linear velocity of 30 cm s^{-1}. Analyses were conducted by using a Shimadzu GC-17A (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) equipped with both flame ionization (FID) and flame thermionic (Model FTD-17) detectors (FTD). Detection was by FID for all analyses used to quantify emissions at different times of the day, and by FTD for three analyses to determine presence of nitrogen in eluted peaks. GC peak areas were measured using Empower™ 2 Chromatography Data Software (Waters Corporation, Milford, MA, USA). Kovats indices were calculated for putative pheromone peaks.

Gas Chromatography–Mass Spectrometry Analyses of Chemicals in Male Volatiles Volatiles were collected by SPME for a 1-h period, 9–10 h after onset of photophase, the time of day when the largest peaks were observed. Chemicals were thermally desorbed from the fiber for 1 min by splitless injection at 250°C, with the injector purged after 1 min. A DB-1 column (60 m, 0.25 mm ID, 0.25 μm film; Agilent), with helium at 26 cm s^{-1} as carrier gas, was used for the analyses. Column oven temperature was programmed from 50°C to 200°C at 5°C min^{-1}. Gas chromatography–mass spectrometry (GC–MS) data were acquired with a Hewlett Packard 6890 GC coupled with an HP 5973 Network Mass Selective Detector [in electron ionization (EI) mode at 70 eV] over a mass range of 20–550 amu. Identification of 2,5-dimethylpyrazine (**1**) (DMP) and 2,3,5-trimethylpyrazine (**3**) (TMP) was by computer matching with spectra in the NIST 98 Library of Mass Spectra and Subsets (Hewlett-Packard) and by GC and MS comparison to commercial samples of 2,3-, 2,5-, and 2,6-dimethylpyrazine and 2,3,5-trimethylpyrazine (Aldrich Chemical Co., Milwaukee, WI, USA). The mass spectrum of the unknown peak that was eventually identified as 3,6-dihydro-2,5-dimethylpyrazine (**2**) (DHDMP) did not match with any spectrum in the NIST 98 Library database. DHDMP in male emissions was identified by using its molecular weight as determined by GC–MS, GC-FTD analysis, and matching its GC retention times and mass spectrum with those of synthetic DHDMP.

Synthesis of DHDMP Oximinoacetone (**6**, Fig. 1) was first prepared, as described by Krems and Spoerri (1947): Ethyl

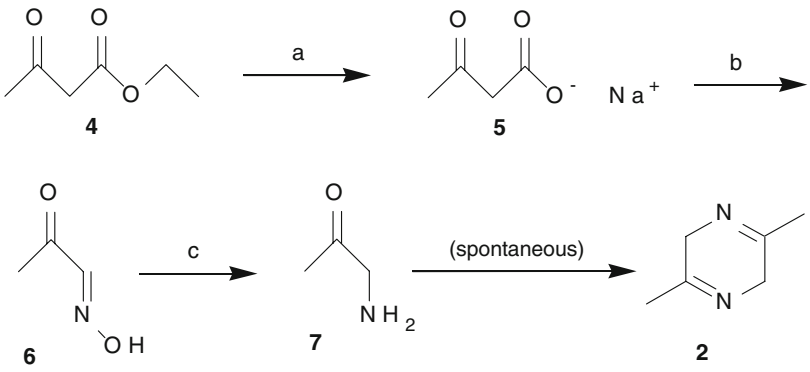
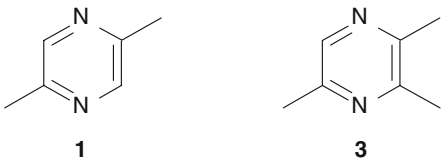


Fig. 1 Synthesis of 3,6-dihydro-2,5-dimethylpyrazine (DHDMP) (**2**). Reaction conditions: *a* 5% aqueous NaOH, 30 min, room temperature; *b* H_2O, HOAc, $NaNO_2$, 0°C to room temperature, 1 h; *c* Al/Hg, tetrahydrofuran, 0°C, 30 min

Other male-specific compounds from *Anastrepha serpentina*



acetoacetate (**4**) (5 g, 0.038 mol) was added to a 5% aqueous solution of NaOH (50 ml, containing 0.063 mol base) and stirred at room temperature for 30 min. The solution, now containing sodium acetoacetate (**5**), was cooled over ice, and acetic acid (7.5 ml) added slowly. A twofold excess of sodium nitrite (5.24 g, 0.076 mol) was added slowly to the acidified solution and stirred for 1 h at room temperature. Oximinoacetone (**6**) was extracted with diethyl ether (25 ml, three times), and the combined extracts dried over anhydrous magnesium sulfate and filtered. Solvent was removed gently by rotary evaporation. EI mass spectra (70 eV) were acquired on a Hewlett Packard 5971A Mass Selective Detector with GC inlet. Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker Avance 500 MHz instrument. One-dimensional ^1H and ^{13}C data are reported, but two-dimensional HSQC and HMBC experiments also were run to aid in assignment of resonances to the proper compounds. Oximinoacetone (**6**): EI-MS: *m/z* (%)= 87 (M^+, 25), 72 (7), 55 (3), 43 (100). ^1H NMR (500 MHz, C_6D_6): 2.31 (s, 3H), 7.92 (s, 1H). ^{13}C NMR (125 MHz, C_6D_6): 24.9, 149.1, 195.6.

Oximinoacetone (**6**) then was reduced to aminoacetone (**7**) with aluminum amalgam as described by Ferris et al. (1968). Aminoacetone dimerized and dehydrated spontaneously to give DHDMP (**2**) (Krems and Spoerri 1947). Aluminum amalgam was prepared by covering aluminum foil (ca. 0.1 g, cut into small pieces) with an aqueous solution of mercuric chloride (5%, 5 ml) for about 1 min, resulting in grayish turbidity in the solution as metallic mercury began to deposit on the foil surface. The solution was decanted off, and the foil quickly rinsed twice with water (5 ml) and two times with tetrahydrofuran (THF, 5 ml). The foil was placed in fresh THF (5 ml) and stirred at ice temperature. Oximinoacetone (**6**) (ca. 0.1 g) was added in one portion. Reaction soon set in, accompanied by the formation of a greenish turbidity in the solution. After 30 min, the solution was transferred to a vial and tightly capped and stored in the freezer. Aliquots were removed and diluted with hexane for GC studies. In addition to compound **2**, the solution contained the air oxidation product, DMP (**1**) and unreacted oximinoacetone (**6**). Purification of **2** was impracticable because it readily oxidizes in air, but the impurities were easily separable from product **2** by GC.

Mass and NMR spectra were acquired for the product mixture as described for oximinoacetone. 3,6-Dihydro-2,5-dimethylpyrazine (**2**): EI-MS: *m/z* (%)=110 (M^+, 34), 95 (1), 69 (100), 54 (19), 42 (89), 41 (48). ^1H NMR (500 MHz, C_6D_6): 1.71 (s, 3H), 3.66 (s, 2H). ^{13}C NMR (125 MHz, C_6D_6): 23.5, 51.6, 166.6. 2,5-Dimethylpyrazine (**1**): EI-MS: *m/z* (%)=108 (M^+, 100), 81 (12), 66(2), 52 (5), 42 (93). ^1H NMR (500 MHz, C_6D_6): 2.26 (s, 3H), 8.15 (s, 1H). ^{13}C NMR (125 MHz, C_6D_6): 20.6, 143.4, 150.5.

Determination of Emission Rates of DMP and DHDMP

Collections of volatiles and GC analyses were conducted as described above. Emission rates were calculated by using the methods and principles found in Bartelt (1997) and Bartelt and Zilkowski (1999). Emission of volatiles from the flies was assumed to be fairly constant during the 1-h sampling periods suggesting that the volatiles in the round bottom flask had achieved a steady rate (i.e., that the volatile concentration within the flask was constant, and thus, that the emission rate from the flies was equivalent to the emission rate through the exit port; Bartelt and Zilkowski 1999). The SPME calibration factor, *K*, for DMP was

previously measured as 11.2 ml (Bartelt 1997), and this value also was used in calculations for DHDMP because the two compounds are of similar volatility. One-hour sampling allowed DMP and DHDMP to equilibrate fully with the SPME fiber (Bartelt 1997), so that their concentrations in the exit air stream were simply proportional to their amounts in the fiber coating (measured by GC). The transfer of sample from the fiber to the GC column was assumed to be quantitative. The response factor for the FID was calibrated by using quantitative standard solutions of DMP. The absolute emission rates were calculated by using the GC-measured amounts, *K*, and the air flow rate past the SPME fiber (Bartelt and Zilkowski 1999).

Statistical Analyses Means and standard errors were calculated for percentages of males calling and mating at various times of the day. Emissions (GC peak areas) of DMP, DHDMP, and TMP from calling males were analyzed to determine the effect of time of day on amount emitted. The effect of time of day was tested by ANOVA by using 1-h time periods as a discrete independent variable. Effect of test day was also partitioned out of the total sum of squares. ANOVAs were conducted by using SuperANOVA (Abacus Concepts 1989).

Results

Diurnal Rhythm of Calling and Mating Wild males began calling within 2 h after sunrise (Fig. 2). Calling behavior of males from Veracruz occurred during most of the photophase, peaking from 9 to 12 h after sunrise. Calling by males from Chiapas followed a similar pattern, but levels were lower and no distinct peak was observed. Laboratory males used in the collection of volatiles also followed a similar pattern (note that no observations were made for this during hours 1–5 and 14 as they were exposed only to 13 h of daylight), with the percentage of males calling generally higher than for the wild insects.

Mating of wild flies from Veracruz followed a pattern similar to calling of laboratory and Veracruz males (Fig. 3). Mating by wild flies from Chiapas appeared to peak earlier in the day. Frequencies of matings were low in these observations, making all comparisons tentative.

Identification of Putative Pheromones in Male Emissions GC analysis indicated that emissions by males varied with time of day for only three compounds. Two of these were tentatively identified as DMP and TMP by computer matching of their mass spectra with spectra in the NIST 98 Library of Mass Spectra and Subsets. The identities of these compounds were confirmed by matching GC retention times and mass spectra with those of authentic compounds. Both produced larger relative responses on the FTD than on the FID, confirming the presence of N bonded to C.

The third chemical was emitted at the highest rate by males, but its mass spectrum (Fig. 4a) did not match any spectra in the library. It also yielded a larger response by the FTD than the FID, indicating the presence of N. Its retention time was longer than that of DMP but close enough to suggest a similar molecular weight (Kovats indices: DMP, 895; unknown, 965). Its probable molecular weight was 110 amu, and the likely molecular formula was $C_6H_{10}N_2$, from isotopic analysis based on the relative abundances of the M+1 ion and the molecular ion, and because an even molecular weight would correspond to an even number of nitrogen atoms. Because the molecular weight was 2 amu greater than the molecular weight of

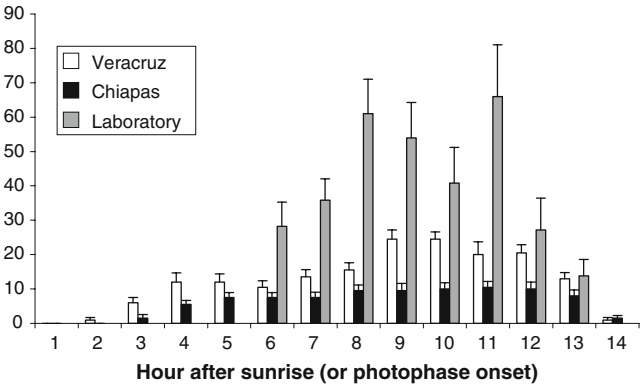


Fig. 2 Mean percentage of male *Anastrepha serpentina,* originating from a laboratory colony and two wild populations (from Veracruz and Chiapas, Mexico), exhibiting calling behavior in the laboratory with respect to time of day. For the laboratory colony, no observations were made during hours 1–5 and 14 (i.e., there were only 13 h of daylight). *Lines atop bars* are SEM

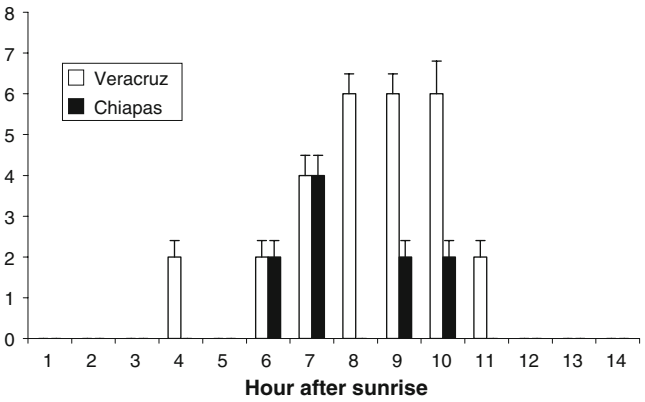


Fig. 3 Mean percentage of matings of *Anastrepha serpentina* from two wild populations (from Veracruz and Chiapas, Mexico) in the laboratory with respect to time of day. *Lines atop bars* are SEM

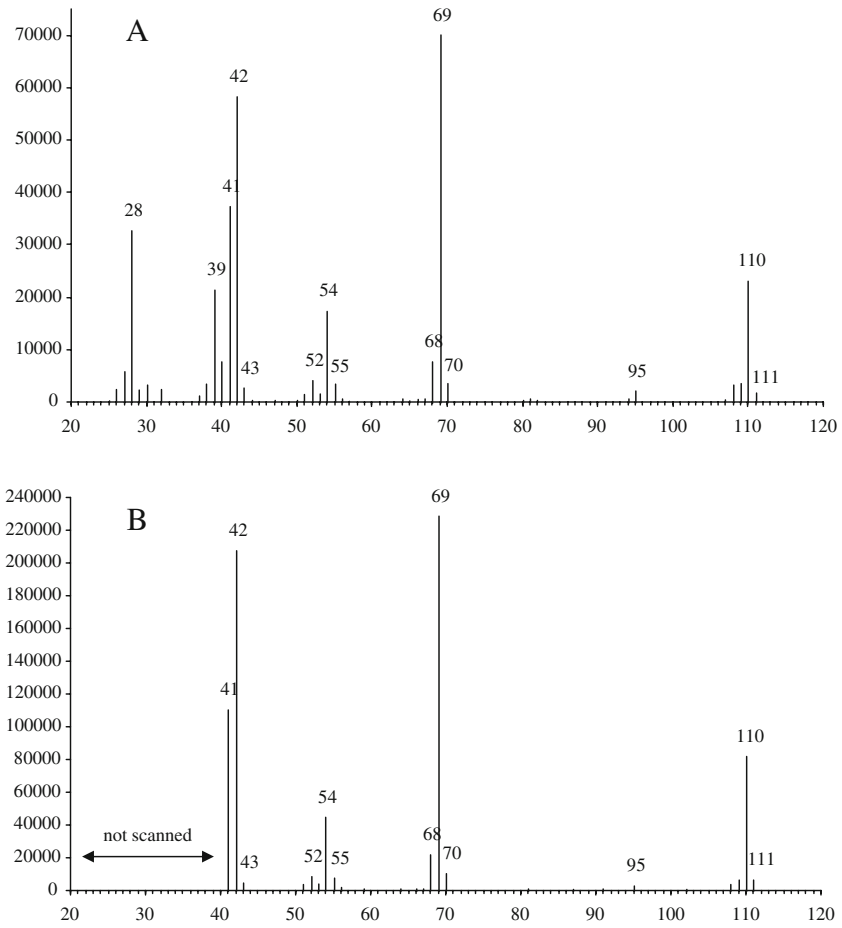


Fig. 4 Electron ionization mass spectra of 3,6-dihydro-2,5-dimethylpyrazine (DHDMP) (**2**). **a** Chemical with Kovats retention index 965 in emissions of calling male *Anastrepha serpentina*. **b** Chemical with Kovats retention index 965 in product of reaction of oximinoacetone (**6**) on aluminum amalgam

DMP and the molecular formula was the same as that of DMP plus two H atoms, the unknown was tentatively identified as a dihydro-dimethylpyrazine, and standards were synthesized to determine exactly which compound it was. Fortuitously, the first standard, 3,6-dihydro-2,5-dimethylpyrazine matched the unknown by mass spectrum (Fig. 4b) and GC retention times, and we concluded that the *A. serpentina* compound was probably DHDMP.

DMP and DHDMP were not found in emissions of females. A small amount of TMP was present in collections from females. The amount was about 2% of the amount found in male emissions, and it could have resulted from imperfect fiber cleaning after a GC determination of male emissions.

Male Emissions vs. Time of Day Emissions of DMP and DHDMP by males in laboratory experiments showed definite peaks about 7–11 h after onset of the 13-h photophase (Fig. 5). Emissions of DMP were significantly greater at 9 and 11 h than at any other time except 10 h (F=4.2; df=10, 53; P< 0.001). Emissions of DHDMP were greater between 9 and 11 h than at any other times (F=5.5; df=10, 53; P<0.001). There were no significant differences with time of day for emission of TMP. A chromatogram of male emissions at 10 h after the onset of photophase is shown in Fig. 6.

Actual emission rates were determined for DMP and DHDMP. Peak emission of DMP was calculated as 1.8 µg/h/male (N=9), 11 h after onset of photophase, and 3.3 µg/h/male (N=9) for DHDMP, 10 h after the onset of photophase.

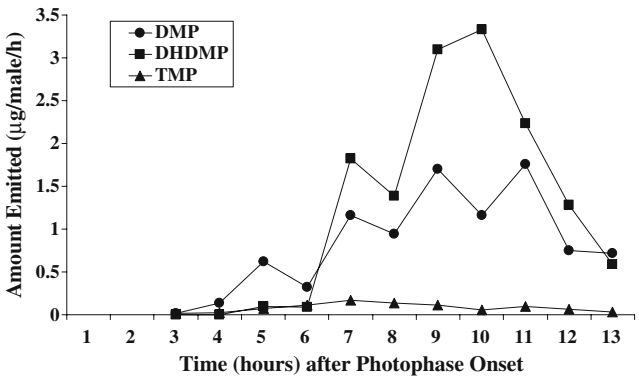


Fig. 5 Emissions of putative pheromone compounds by male *Anastrepha serpentina* with respect to time of day. (**1**) 2,5-dimethylpyrazine (*DMP*); (**2**) 3,6-dihydro-2,5-dimethylpyrazine (*DHDMP*); (**3**) 2,3,5-trimethylpyrazine (*TMP*)

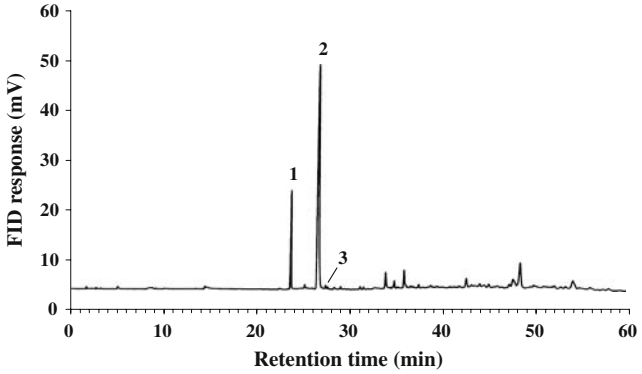


Fig. 6 Gas chromatogram (flame ionization detection) of emissions of calling male *Anastrepha serpentina*. (**1**) 2,5-dimethylpyrazine; (**2**) 3,6-dihydro-2,5-dimethylpyrazine; (**3**) 2,3,5-trimethylpyrazine

Discussion

Evidence for the most abundant compound emitted by male *A. serpentina* being DHDMP is strong. The natural and synthetic compounds were identical by GC retention, mass spectrum, and responses on the FTD. It is unlikely that other isomers (with respect to methyl position, nitrogen position, or double-bond position) would match all these criteria. Furthermore, the synthetic scheme (head-to-tail dimerization of oximinoacetone), the NMR spectra, and presence of DMP as a reaction by-product (due to oxidation of DHDMP in air) are entirely consistent with the desired isomer of DHDMP that was made. NMR of the natural compound would have given further confirmation of structure, but this was not feasible because the lability of DHDMP would make it difficult to collect and purify enough material. After a thorough search of the literature, we conclude this to be the first report of DHDMP as a natural product.

The identifications of the less abundant, fly-derived compounds, DMP and TMP, were also based on MS and GC comparison to synthetic standards. These were considered unequivocal because double-bond position was not an issue, and because standards were available for all of the possible methyl group positions.

In this study, males used for the collection of emissions were fed a combination of sugar and protein. Aluja et al. (2001) reported that male-mating performance was influenced strongly by adult diet. For example, *A. serpentina* males with varying adult feeding histories (adults fed from the time of emergence on dry sucrose, open fruit, a mixture [3:1] of dry sucrose and hydrolyzed protein, or a mixture of dry sucrose and bird feces) that were competing for access to females in a field cage, exhibited greatly differing rates of copulatory success. Those with access only to sucrose never mated, while those with access to the sucrose/protein mixture mated the most (greatest proportion) and were able to mate repeatedly over a 3-day period. The latter is pertinent, as it has been shown that adult diet influences the composition of the sex pheromone of *A. suspensa* (Epsky and Heath 1993). In the case of *A. serpentina*, in which all of the putative pheromone components contain nitrogen, the need for protein in order for males to produce these chemicals in the high quantities reported above seems especially important.

The presence of pyrazines in male emissions of *A. serpentina* has precedence in Tephritidae. Baker et al. (1985) stated that they found nine components of male Mediterranean fruit fly emissions, including 2-ethyl-3,5-dimethylpyrazine, although no chemical data were given, and this compound was not tested for biological activity. Jang et al. (1989) found 2,5-dimethyl-3-ethylpyrazine as a trace component of male emissions of this fly. This compound elicited a small electroantennogram response from female antennae. Baker et al. (1990) identified 2,3-dimethylpyrazine and DMP in anal glands of Mediterranean fruit fly. These pyrazines were attractive in field tests, but the tests did not determine if the response was sexual attraction. However, Flath et al. (1993) did not find pyrazines in emissions of male Mediterranean fruit flies. In the case of the Mexican fruit fly, sexually inactive males and females are attracted to both DMP (Robacker and Flath 1995) and TMP (Robacker and Bartelt 1997).

De Lima et al. (1996) indicated that pyrazines had been detected as pheromone components in *Bactrocera dorsalis*, but gave no specific reference. Baker et al. (1982) identified methylpyrazine, TMP, and tetramethylpyrazine from rectal glands of melon fly (*Bactrocera cucurbitae*), but Nishida et al. (1990) did not find these chemicals in melon fly, possibly due to strain differences. However, Perkins et al. (1990) reported that Lewis (1987) confirmed the presence of TMP and tetramethylpyrazine but could not find methylpyrazine.

Chuman et al. (1987) identified 2-methyl-6-vinylpyrazine as the sex pheromone of the papaya fruit fly (*Toxotrypana curvicauda*), a species closely related to *Anastrepha*. De Lima et al. (1996) found DMP, TMP, 3-ethyl-2,5-dimethylpyrazine, and 3-butyl-2,5-dimethylpyrazine in salivary glands of male *A. fraterculus*. These chemicals were not tested for pheromonal activity. Calling males of other species in the *fraterculus* group, including *A. suspensa*, *A. obliqua*, and *A. ludens*, are not known to emit pyrazines (Heath et al. 2000).

DHDMP is in a class of compounds called dihydropyrazines that have not been reported as chemicals emitted by calling Tephritidae. Moreover, DHDMP has never been reported in nature. While the structurally related pyrazines are generally stable, dihydropyrazines are not. The stability of pyrazines results from the aromatic nature of the six-

membered ring (analogous to benzene, but with N in place of C at the 1 and 4 positions). The instability of dihydropyrazines, in general (Krems and Spoerri 1947), and of DHDMP in particular (Wilen 1970), follows from the tendency to lose two hydrogens and aromatize. Even contact with air can cause this conversion. As shown in this work, DHDMP is stable enough to be released from living flies and to be captured by an SPME fiber, but it is apparently not stable enough to survive protracted or relatively harsh volatile collection procedures.

One of the significant implications of this work is that dihydropyrazines might be common in insects, perhaps serving as pheromones or pheromone precursors, but have never been found because of the methodology constraints discussed above. If this is the case, then it is also possible that the pyrazines found in this work, and in other Tephritidae (see above), actually may not be produced by the flies but are present only in male emissions as artifacts of the breakdown of dihydropyrazines.

As a possible consequence of the instability of dihydropyrazines, Heath et al. (2000) reported no chemicals associated with calling behavior of male *A. serpentina*. These authors collected male-emitted chemicals by passing male emissions through Porapak Q, an adsorbent that has been used extensively by numerous insect chemical ecologists to identify pheromones and other semiochemicals. Apparently, SPME PDMS used in the present work was better suited than Porapak Q for collecting emissions of *A. serpentina*, at least in part because of the lability of DHDMP. The handling of DHDMP, including collection on Porapak Q, solvent extraction, sample focusing by solvent evaporation, and storage, likely resulted in nearly complete breakdown of DHDMP. Why Heath et al. (2000) did not report finding DMP and TMP is difficult to explain, but may be a function of affinities of PDMS and Porapak Q for pyrazines.

Because these compounds (**1**–**3**) are emitted in the greatest amounts at the time of day when calling and mating of flies are greatest, we suggest that they may be sex pheromone components. Testing of the behavioral activity elicited by these compounds will be a topic of future research.

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Aggregation Pheromone of the Qinghai Spruce Bark Beetle, *Ips nitidus* Eggers

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Abstract Volatiles from hindgut extracts of males of the Qinghai spruce bark beetle, *Ips nitidus*, from different attack phases (phase 1: unpaired males and phases 2–4: males joined with one to three females) and hindgut extracts of mated females were analyzed by gas chromatography–mass spectrometry (GC–MS)/flame ionization detection (FID) with both polar and enantioselective columns. The GC–MS/FID analyses demonstrated that unpaired males from attack phase 1 (nuptial chamber constructed) produced 2-methyl-3-buten-2-ol, approx. 74%-(−)-ipsdienol, and (−)-*cis*-verbenol as major hindgut components, and (−)-*trans*-verbenol, (−)-ipsenol, (−)-verbenone, myrtenol, and 2-phenylethanol as minor or trace components. The quantities of 2-methyl-3-buten-2-ol and especially ipsdienol decreased after mating during phases 2–4, whereas the quantities of (−)-*cis*- and (−)-*trans*-verbenol did not change. In contrast, the quantity of (−)-ipsenol seemed to increase as mating activity progressed. After mating with three females (harem size=3; phase 4), only trace to small amounts of male-specific compounds were detected from *I. nitidus* male hindguts. Chemical analysis of the hindgut extracts of mated females showed only trace amounts of semiochemicals. A field-trapping bioassay in Qinghai, China showed that the four-component "full blend" containing the three major components, 2-methyl-3-buten-2-ol, (±)-ipsdienol, and (−)-*cis*-verbenol, plus a minor component, (−)-*trans*-verbenol, caught significantly more *I. nitidus* (♂/♀=1:2.2) than did the unbaited control and two binary blends. The replacement of (±)-ipsdienol with nearly enantiomerically pure (−)-ipsdienol in the "full blend" significantly reduced trap catches, which suggests that both enantiomers are needed for attraction. On the other hand, removal of (−)-*trans*-verbenol from the active "full blend" had no significant effect on trap catches. Our results suggest that the three major components, 2-methyl-3-buten-2-ol, 74%-(−)-ipsdienol, and (−)-*cis*-verbenol (at 7:2:1), produced by unpaired fed males, are likely the aggregation pheromone components of *I. nitidus*, thus representing the first characterization of an aggregation pheromone system of a bark beetle native solely to China.

Keywords Attractant · Coleoptera · Enantiomeric composition · GC–MS · *Ips nitidus* · Ipsdienol · 2-Methyl-3-buten-2-ol · *Picea* · Scolytidae · Semiochemical · Trap · *cis*- and *trans*-Verbenol

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Introduction

The Qinghai spruce bark beetle, *Ips nitidus* Eggers (Coleoptera: Scolytidae), was first described in 1933 from specimens collected by Eggers from Muke Tatslenlu (Kangding), Sichuan Province, China. Fu (1983) recorded

I. nitidus from several species of spruce (*Picea*) in five western provinces of China (Gansu, Qinghai, Sichuan, Xinjiang, and Yunnan). In the 1970s, *I. nitidus* was recognized as one of the dominant bark beetle species in Qinghai spruce forests [*Picea crassifolia* (Kom.)] of the Qilian Mountain, Gansu Province (Fu 1983). This bark beetle infests weakened, wind-thrown, or burned trees, and at high population densities, it attacks healthy spruce trees. Recently, *I. nitidus* together with two other newly described sympatric bark beetles, *Ips shangrila* Cognato and Sun (Cognato and Sun 2007) and *Pseudips orientalis* (Wood & Yin) (Cognato 2000), were identified as the most destructive forest pest insects in the Maixiu Forest Park of Qinghai Province, China; where, since 2001, their outbreaks (independent or mixed) have caused significant tree mortality both in plantations and natural stands of *P. crassifolia* (Xue et al. 2003; Liu et al. 2007, 2008).

During the outbreaks in 2001–2005, the species was mis-identified as *Ips typographus* (L.) by Chinese entomologists (Xue et al. 2003), but in 2006, it was correctly identified by Milos Knížek (Forestry and Game Management Research Institute, Czech Republic) as *I. nitidus*. The basic biology and host colonization behavior of this species in natural spruce forests were recently described (Liu et al. 2007, 2008). First attempts to catch adult beetles with traps baited with *I. typographus* pheromone lures during their dispersal flights were made in the mid 1980s by Zhou et al. (1995) in Gansu Province with some positive results (assuming the captured beetle species was correctly identified). Additional bouts of field screening of potential attractants (for future monitoring or mass-trapping operations) with the known *Ips* aggregation pheromone components and their various combinations have been undertaken by local forest departments in recent years, but these efforts have yielded little success.

In contrast to the previous focus on screening semiochemicals by evaluating flight responses, our objectives were to (1) identify the aggregation pheromone of *I. nitidus*, (2) analyze the quantitative variation of pheromone components from different attack phases, (3) determine the enantiomeric compositions of major chiral pheromone components, and (4) test the behavioral activity of the key male-specific compounds as flight attractants in the field in Qinghai, China.

Methods and Materials

Collection and Preparation of Samples Live adult *I. nitidus* of different subcortical attack phases [phase 1: unpaired male in nuptial chamber; phase 2: one mated male with one female; phase 3: one mated male with two females; and phase 4: one mated male with three females in the galleries] were collected between 22 and 25 May 2008 from two naturally attacked wind-thrown Qinghai spruce trees [*P. crassifolia*)] at Maixiu Forest Park (35°08′–35°30′ N; 101° 33′–102°03′ E; ca. 2,900–3,000 m elevation), Huangnan Tibetan District, Qinghai Province, China. Beetles from the same family gallery system were placed in a 2-ml polyethylene centrifuge tube (Fisher Scientific, Pittsburgh, PA, USA) and immediately put into an outdoor cooler (ca. 4°C). The centrifuge tubes were separated into categories of attack phases on the same day of collection in the laboratory, and the hindguts were dissected quickly. Sexes were distinguished based on their elytral spine differences (Third elytral spine of males is much larger and more strongly capitate than the other three spines, whereas in females there are no obvious differences among the second, third, and fourth spines; Song et al. unpublished data) and presence of the aedeagus (male) or eggs (female) in cases where the spines were damaged. Male hindguts from the same attack phase and the same sample tree were extracted immediately with 1 ml redistilled pentane (with 2 μg of heptyl acetate as internal standard) in a 2-ml amber glass vial. Female hindguts from various attack phases and both sample trees were pooled and extracted in the same fashion as the male hindguts. Hindgut extracts (four to 27 guts/sample for males from different attack phases and 19 guts/sample for mated females) were shipped to the USA by express mail and kept at −20°C until analyzed chemically [gas chromatography with mass spectrometric detection (GC–MS) and gas chromatography with flame ionization detection (GC–FID)].

GC–MS Analysis All hindgut samples were analyzed on a combined Agilent 6890N gas chromatograph (GC) and an Agilent 5973N mass selective detector equipped with a polar column (INNOWax; 60 m×0.25 mm×0.5 μm film thickness; Agilent Technologies, Wilmington, DE, USA). The GC oven was programmed at 50°C for 1 min, with a rise to 230°C at a rate of 10°C/min, and held at 230°C for 25 min. Injector and transfer line temperatures were both 250°C. Helium was used as carrier gas at a constant flow of 26 cm/s. Compounds were identified by comparison of retention times and mass spectra with those of authentic standards (see Chemical Standards below). All of the analytes are previously known bark beetle semiochemicals.

GC–FID Analysis Hindgut samples also were injected into a Varian CP-3800 GC equipped with a polar column (INNOWax; 30 m×0.53 mm×1.0 μm film thickness; Agilent Technologies, Wilmington, DE, USA) and FID for compound quantification based on the internal standard (IS 2 μg of heptyl acetate in each sample; assuming similar or identical response factors between the analytes and the IS). Helium was used as the carrier gas at a constant flow of

26 cm/s, and the injector and detector temperatures were 220°C and 300°C, respectively. Column temperature was 50°C for 1 min, rising to 240°C at 10°C/min, and then held for 10 min.

Enantioselective GC–FID The enantiomeric analyses of male hindgut extracts (from phases 1 to 2), and a synthetic mixture of several key *Ips* pheromone compounds including (±)-ipsenol, (±)-ipsdienol, (1*S*,2*S*)-(−)-*cis*-verbenol, (1*S*,2*R*)-(−)-*trans*-verbenol, amitinol, (1*S*)-(−)-verbenone, and *E*-myrcenol (50 ng/μl each in hexane) were conducted by injecting the samples splitless on a Varian CP-3800 GC equipped with an Rt-bDEXm™ column (30 m×0.25 mm×0.25 μm film thickness; Restek, Bellefonte, PA, USA). Helium was used as carrier gas, and the injector/detector temperatures were both 230°C. Column temperature was 80°C for 1 min and rose to 200°C at 2°C/min. Elution orders of the (−)- or (+)-enantiomers of ipsenol and ipsdienol [(−)- eluted before (+)- for both compounds] were determined by injecting SPME (CAR/PDMS, 75 μm; Supelco, Bellefonte, PA, USA) samples of synthetic 97%-(+)-ipsdienol and 97%-(−)-ipsenol onto the same column, which confirmed the previous reports with a similar stationary phase (Seybold et al. 1995b; Macías-Sámano et al. 1997; Savoie et al. 1998). Since no pure (+)-enantiomers of *cis*- or *trans*-verbenol, and verbenone were available for comparison, our suggestions on their enantiomeric assignments were based entirely on the retention time matches to the synthetic (−)-enantiomers. Therefore, these assignments should be considered provisionary.

Chemical Standards Synthetic compounds were obtained from various commercial and noncommercial sources: (±)-ipsenol (95%, chemical purity: cp), (±)-ipsdienol (95% cp), (−)-*cis*-verbenol (98% cp, unknown enantiomeric purity-ep), and (−)-verbenone (99% cp, unknown ep; Bedoukian Research Inc., Danbury, CT, USA); amitinol (98% cp, W. Francke, Universität Hamburg, Hamburg, Germany); *E*-myrcenol (95.2% cp, SciTech, Prague, Czech Republic); 2-methyl-3-buten-2-ol (97% cp, Acros, Morris Plains, NJ, USA); (−)-ipsenol (97.3% cp; 97.5% ep), (+)-ipsdienol (96% cp; 98.2% ep), (−)-ipsdienol (95% cp; 97% ep) and (−)-*trans*-verbenol (>95% cp, unknown ep) [Pherotech (now Contech) International, Inc., Delta, BC, Canada]; heptyl acetate (>98% cp, food grade) and (±)-1,3-butanediol (99% cp) (Sigma-Aldrich, St. Louis, MO, USA).

Field Trapping To assay for behavioral activity of the potential semiochemicals, a field-trapping experiment was carried out from 30 July to 24 August 2008 at the Maixiu Forest Park, which is the same park from where the *I. nitidus* hindgut samples were collected. Three sets of cross-barrier type traps (Sino-Czech Trading Co. Ltd. Beijing, China; see photo insert in Fig. 3) were set up along the edge of a *P. crassifolia* forest stand on a northern slope next to a creek at Douheyan, with >30 m between trap sets and ca. 10 m between traps within each set, and >10 m from the nearest trees. Within each set, eight traps were baited with different blends (full or partial blends; racemic or enantiomerically pure) of the key male-produced volatile compounds in their natural production ratios; a ninth trap was left unbaited as a negative control (Table 1). Release rates of the potential semiochemicals varied from <0.3 to 30 mg/day (Table 1). The positions of traps together with dispensers within each set were assigned randomly, and to minimize any positional effects, the dispensers' positions were re-

Table 1 Semiochemical treatments and dispensers for field-trapping experiment with the Qinghai spruce bark beetle, *Ips nitidus*, Qinghai Province, China, 30 July–24 August, 2008

Chemical	Treatments and loading (mg/dispenser)[a]								
	A	B	C	D	E	F	G	H	I
2-Methy-3-buten-2-ol[b]	2,000	2,000	2,000	2,000	2,000	2,000	2,000	2,000	0
(−)-*cis*-Verbenol[c]	15	15	15	15	0	0	0	0	0
(−)-Ipsdienol (97%) [c]	30	0	30	0	30	0	30	0	0
(±)-Ipsdienol [c]	0	30	0	30	0	30	0	30	0
(−)-*trans*-Verbenol [c]	7	7	0	0	7	7	0	0	0
1,3-Butanediol (inert solvent)	750	750	750	750	750	750	750	750	750

[a] Loading ratios were based on the natural ratios of these chemicals produced by unpaired males from phase 1 (Table 2)

[b] 2-Methyl-3-buten-2-ol alone was released from a 2-mil polyethylene bag (7×5 cm) with felt. Release rate was 30 mg/day measured at 22°C in a laboratory fumehood

[c] These semiochemicals (individuals: G/H or in combinations: A/B/C/D/E/F) were dissolved in 750 ml of 1,3-butanediol (as an inert solvent) and loaded in a 2-mil polyethylene bag (3×5 cm) with felt. The exact release rates of these chemicals were not determined with normal gravimetric methods due to the hygroscopic nature of the inert solvent. Estimated release rates by solid phase microextraction (SPME) followed by GC analysis indicated a low overall release rate (<0.3 mg/day) for individual components or mixtures of ipsdienol, and *cis*- and *trans*-verbenol

randomized after each replicate (Byers 1991) when >10 beetles were caught in the traps with the most attractive bait.

Statistical Analyses Trap catch data were converted to proportion (P) of total captured beetles within each replicate. Data then were transformed by arcsin$\sqrt{P}$ to meet the assumptions of normality and homogeneity of variances for ANOVA. Means were compared by ANOVA followed by the Ryan–Einot–Gabriel–Welsh (REGW) multiple Q test (SPSS 16.0 for Windows) at α=0.05 (Day and Quinn 1989). Due to the zero responses to the unbaited control and the two binary blends, these three treatments were not included in the ANOVA and range tests. Trap catch data (untransformed) were also analyzed with the nonparametric Kruskal–Wallis ANOVA on ranks followed by the Student–Newman–Keuls test to separate means of the treatments with and without zero catches (Zar 1984).

Results

Chemical Analysis Eleven volatile compounds from the extracts of male hindguts were identified by GC–MS and quantified by GC–FID. These compounds included 2-methyl-3-buten-2-ol, ipsdienol, and *cis*-verbenol (at 7:2:1) as major components, and *trans*-verbenol, ipsenol, and verbenone as minor or trace components (Fig. 1, Table 2). Myrtenol and 2-phenylethanol, found in several *Ips* beetles (Zhang et al. 2000), but not confirmed as pheromone components, also were detected as minor components (Fig. 1, Table 2). 3-Hydroxy-2-butanone, 1-heptanol, and isobutyric acid were present in all samples.

GC–FID analysis with an enantioselective stationary phase of the hindgut extracts of the *I. nitidus* males from phases 1 to 2 and a synthetic mixture of *Ips* pheromone compounds showed that *I. nitidus* males produced 74±3%-(−)-ipsdienol (mean±SE; N=4; Fig. 2). The predominant enantiomers of other chiral compounds were tentatively determined as (−)-*cis*- and (−)-*trans*-verbenol, (−)-verbenone, and likely (−)-ipsenol (Fig. 2).

Mean amounts (N=2) of the three major components (2-methyl-3-buten-2-ol, ipsdienol, and *cis*-verbenol) from phase 1 were 2,800, 760, and 330 ng/male (ca. 7:2:1 ratio), respectively, which are 2–100 times higher than the three minor components (*trans*-verbenol, ipsenol, and verbenone; Fig. 1, Table 2). Although no statistical analysis was performed because of the limited data set, the quantities of 2-methyl-3-buten-2-ol and especially ipsdienol decreased after mating (i.e., during phases 2–4), whereas the quantities of *cis*- and *trans*-verbenol, myrtenol, and 2-phenylethanol showed no obvious changes (Fig. 1, Table 2).

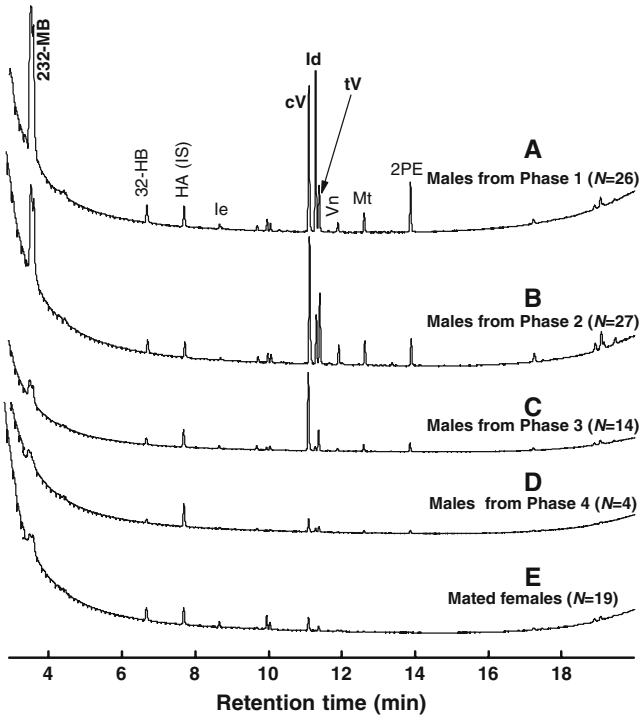

Fig. 1 Representative gas chromatograms (polar column with FID detection) of compounds in hindgut extracts of male *Ips nitidus* from different attack phases (*A–D*) and mated females from mixed phases (*E*). Heptyl acetate (HA; 2 µg/sample) was added as an internal standard to the hindgut extracts. Analytes included 2-methyl-3-buten-2-ol (*232-MB*), *cis*-verbenol (*cV*), ipsdienol (*Id*), ipsenol (*Ie*), *trans*-verbenol (*tV*), (−)-verbenone (*Vn*), 3-hydroxy-2-butanone (*32-HB*), myrtenol (*Mt*), and 2-phenylethanol (*2PE*)

Interestingly, the quantity of ipsenol seemed to increase as the mating activity progressed (Table 2).

In the hindgut extracts of mated females (taken from mixed phases), only trace amounts of male *Ips*-related semiochemicals were detected by GC–MS or GC–FID (Fig. 1 E). These traces may have been due to contamination in the gallery from semiochemical-producing males. In female extracts, we detected quantities of 3-hydroxy-2-butanone, 1-heptanol, and isobutyric acid that were similar to those in male extracts (Table 2).

Field-Trapping Experiment Due to extremely low populations of *I. nitidus* sister broods and heavy winds and rains during the period of our field study (late July to mid August), only six replicates were obtained from two sets of traps (sets 2–3). Traps and dispensers in set 1 were damaged by a flood or partially missing, thus giving no valid data. The unbaited control traps and traps baited with the binary blends (Table 1, treatments G and H): 2-methyl-3-buten-2-ol plus (±)-ipsdienol or (−)-ipsdienol, did not catch any beetles (Fig. 3). Ternary blends with 97%-(−)-ipsdienol caught only a few beetles and seemed to be less attractive than ternary or quaternary blends with (±)-

Table 2 Quantities of potential semiochemicals (in nanogram) identified from hindgut extracts of *Ips nitidus* males/females of different attack phases, Qinghai Province, China, May 22–25, 2008

Retention time (min)	Chemical	Male hindguts from different attack phases								Mated ♀ hindguts (*N*=1; *n*=19) ng/♀
		Phase 1		Phase 2		Phase 3		Phase 4		
		1♂ (*N*=2; *n*=16–19)		1♂1♀ (*N*=1; *n*=27)		1♂2♀ (*N*=2; *n*=8–14)		1♂3♀ (*N*=2; *n*=4–5)		
		ng/♂	%	ng/♂	%	ng/♂	%	ng/♂	%	
3:28	2-Methy-3-buten-2-ol	2816.5	61.9	1060.6	42.5	844.5	41.3	924.3	47.1	trace
6:39	3-Hydroxy-2-butanone	82.9	1.8	69.5	2.8	88.6	4.3	104.2	5.3	107.0
8:39	1-Heptanol	39.4	0.9	19.3	0.8	38.0	1.9	61.6	3.1	45.0
9:41	(−)-Ipsenol	9.5	0.2	37.2	1.5	60.9	3.0	112.1	5.7	
10:02	Isobutyric acid	49.2	1.1	59.8	2.4	72.9	3.6	140.9	7.2	89.0
11:05	(−)-*cis*-Verbenol	332.5	7.3	507.2	20.3	549.0	26.9	219.4	11.2	
11:16	74%-(−)-Ipsdienol	760.7	16.7	183.0	7.3	47.0	2.3	71.9	3.7	trace
11:22	(−)-*trans*-Verbenol	163.8	3.6	265.8	10.7	164.2	8.0	137.7	7.0	trace
11:53	(−)-Verbenone	83.5	1.8	76.7	3.1	34.0	1.7	20.2	1.0	
12:35	Myrtenol	73.6	1.6	93.9	3.8	66.3	3.2	76.6	3.9	
13:51	2-Phenylethanol	142.1	3.1	119.8	4.8	77.5	3.8	91.9	4.7	

N number of hindgut extracts per attack phase, *n* number of hindguts per extract

ipsdienol (Fig. 3). The four-compoment "full blend" containing 2-methyl-3-buten-2-ol, (±)-ipsdienol, (−)-*cis*-verbenol, and (−)-*trans*-verbenol, caught the most beetles. This response was significantly higher than that to the quaternary blend that contained 97%-(−)-ipsdienol (Table 1, treatment A, Fig. 3). Subtraction of (−)-*trans*-verbenol from the most active "full blend" [containing (±)-ipsdienol] slightly reduced trap catches, but this response was not significantly different from that to the quaternary blend (Fig. 3). The sex ratio of captured beetles was estimated as 1:2.2 (♂/♀), based on the pooled sub-samples.

Discussion

This is the first chemical and behavioral analysis of the aggregation pheromone system of the Qinghai spruce bark beetle, *I. nitidus*. GC–MS and GC–FID results demonstrate that males from attack phase 1 (nuptial chamber constructed, unpaired) produced 2-methyl-3-buten-2-ol, ipsdienol, and *cis*-verbenol as major hindgut components, and *trans*-verbenol, ipsenol, and verbenone as minor or trace components (Fig. 1 A, Table 2). Other compounds frequently found in many *Ips*, but not confirmed as pheromone components,

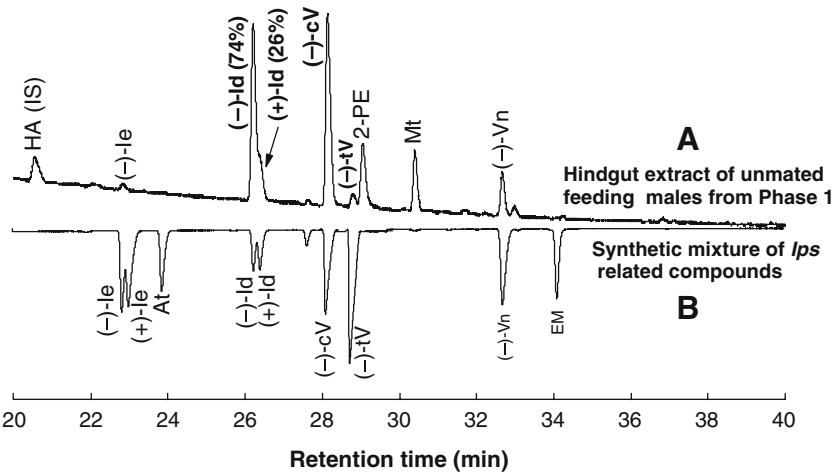

Fig. 2 Enantioselective GC–FID analyses (Rt-bDEXm™ column) of *A* compounds in a hindgut extract of unpaired male *Ips nitidus* from attack phase 1 and *B* a synthetic mixture of *Ips*-related compounds, including (±)-ipsenol (*Ie*), (±)-ipsdienol (*Id*), amitinol (*At*), (−)-*cis*-verbenol (*cV*), (−)-*trans*-verbenol (*tV*), (−)-verbenone (*Vn*), and (*E*)-myrcenol (*EM*; ca. 50 ng/each compound). Amitinol and *E*-myrcenol are achiral. Heptyl acetate (HA; 2 μg/sample) was added as an internal standard to the hindgut extract

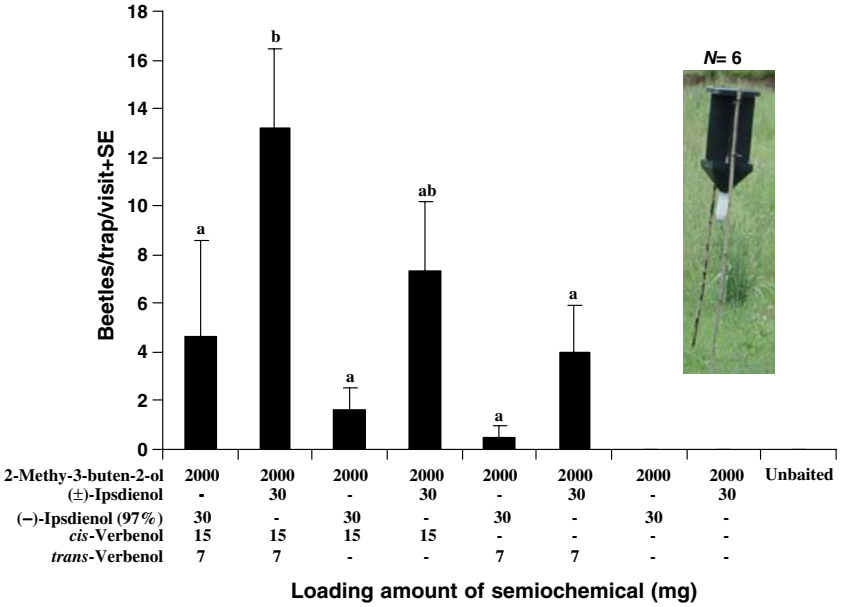

Fig. 3 Mean captures (N=6) of *Ips nitidus* in cross-barrier traps (*photo insert*) baited with different combinations (Table 1) of the key male-produced volatile compounds in their natural production ratios, July 30 to August 24, 2008, Douheyan, Maixiu Forest Park, Qinghai, China. An unbaited trap served as the negative control. *Bars with the same letter* are not significantly different (P>0.05) by REGW multiple Q test after ANOVA on the arcsin$\sqrt{P}$ transformed data of the relative catches, i.e., proportion (P) of total captured beetles within each replicate. Due to the zero responses to the unbaited trap and the two binary blends, these three treatments were not included in the ANOVA and range tests, but were further compared by using the nonparametric Kruskal–Wallis ANOVA on ranks followed by Student–Newman–Keuls test to separate means of the treatments with and without zero catches

such as myrtenol and 2-phenylethanol, also were detected as minor components (Birgersson et al. 1984; Zhang et al. 2000, 2007; Fig. 1, Table 2). 3-Hydroxy-2-butanone, 1-heptanol, and isobutyric acid, commonly related to insect fat tissues (Zhang et al. 2006), were present in all samples, and presumably are not part of the male-produced aggregation pheromone system. Chemical analysis of the hindgut extracts of mated females showed trace quantities of these semiochemicals (Fig. 1 E, Table 2), in agreement with the preponderance of studies that indicate that female *Ips* do not produce behaviorally relevant amounts of aggregation pheromones during attacks (Wood 1982; Byers 1989b).

GC–FID analyses indicated a large variation in the quantities of male-produced volatiles during different attack phases. The maximum production of the major components occurred in phases 1–2, when the nuptial chamber was finished, or only one female was accepted (Fig. 1 A, B). Mating reduced production of the male-specific major hindgut volatiles, especially for 2-methyl-3-buten-2-ol and ipsdienol, whereas the quantities of ipsenol appeared to increase as colonization progressed (Table 2). This might indicate a potential repellent effect of ipsenol during the development of attacks, as reported for *I. typographus* (Schlyter et al. 1992). After mating with three females (harem size=3; phase 4), only trace to tiny amounts of male-specific compounds were detected in *I. nitidus* male hindgut extracts, suggesting that males in this phase may not be capable of attracting females or other males (Fig. 1 D).

Enantioselective GC analysis indicated that *I. nitidus* males produced approx. 74%-(−)-ipsdienol (Fig. 2). This enantiomeric composition is different from those reported for other Eurasian *Ips* spp. (Kohnle et al. 1988, 1991; Zhang et al. 2007), including its sibling species *I. typographus* [95%-(−)] (Kohnle et al. 1991) and the sympatric *I. shangrila* [99%-(+)] and *P. orientalis* [67%-(+)] (Zhang et al., unpublished). However, this enantiomeric ratio is similar to that of eastern populations of North American *Ips pini* (Seybold et al. 1995a). Other chiral compounds were tentatively determined as follows: (−)-*cis*- and (−)-*trans*-verbenol, (−)-verbenone, and likely (−)-ipsenol, based on the retention time comparison with the synthetic standards (Fig. 2), which are similar to the reported absolute configurations for many *Ips* bark beetles (Kohnle et al. 1988).

Our field-trapping data showed that the four-component "full blend" containing the three major components, 2-methyl-3-buten-2-ol, (±)-ipsdienol, and (−)-*cis*-verbenol, plus a minor component, (−)-*trans*-verbenol, caught significant numbers of *I. nitidus* (both females and males; Fig. 3). Replacing (±)-ipsdienol with 97%-(−)-ipsdienol in the "full blend" significantly reduced trap catches, indicating that both enantiomers of ipsdienol might be needed for attraction. This response preference to both enantiomers is

supported by a naturally occurring blend of 74%-(−)-ipsdienol extracted from male hindgut tissue (from phases 1 to 2; Fig. 2). It remains unknown if the natural ratio of ipsdienol enantiomers will prove superior to racemic ipsdienol. Subtraction of (−)-*trans*-verbenol from the active "full blend" (Byers 1992) had no significant effect on trap catches. In fact, this compound also was detected in other Eurasian *Ips* species as a minor component, such as *I. typographus* (Birgersson et al. 1984), and *I. duplicatus* (Byers et al. 1990; Schlyter et al. 1992; Zhang et al. 2007), but was not considered to be part of their aggregation pheromone systems (Schlyter et al. 1987).

I. nitidus and *I. typographus* form a monophyletic group and are sibling species (Cognato and Sun 2007). The proposed aggregation pheromone for *I. nitidus* contains three components: 2-methyl-3-buten-2-ol, ipsdienol, and *cis*-verbenol, whereas the aggregation pheromone system for *I. typographus* contains two components: 2-methyl-3-buten-2-ol and *cis*-verbenol (Kohnle et al. 1988). Ipsdienol is produced as a minor component by mated male *I. typographus* during late attack phases (Birgersson et al. 1984), but has not been considered as part of its aggregation pheromone system (Schlyter et al. 1987; Kohnle et al. 1988). The enantiomeric compositions of ipsdienol isolated from the two species differ [95%-(−) for *I. typographus* (Kohnle et al. 1991) and 74%-(−) for *I. nitidus* (Fig. 2)]. Any differences in the enantiomeric compositions of *cis*-verbenol from these two species have not been reported. Thus, there appears to be some level of disparity in the aggregation pheromone attractants for *I. nitidus* and *I. typographus* that may allow these species to maintain their reproductive isolation within their zone of sympatry (e.g., native spruce forests in Qinghai and Gansu Provinces, China). These differences in the pheromone systems may not be as pronounced as predicted by the saltational evolution hypothesis proposed by Symonds and Elgar (2004), i.e., within certain phylogenetic constraints, pheromone evolution in bark beetles is characterized by large saltational shifts, resulting in substantial phenotypic (i.e., pheromonal composition) differences between sibling species. In fact, this hypothesis is not supported either by experimental data on cross-attraction in the field between two sympatric sibling *Ips* species, *Ips hoppingi* and *Ips confusus*, in the southwestern USA (Cane et al. 1990) or by data on laboratory responses to pheromones by 17 species of *Ips* (Lanier and Wood 1975). *I. nitidus* and two other sympatric bark beetles, *I. shangrila* and *P. orientalis*, share a common or similar spatial and temporal niche(s) in *P. crassifolia* (Liu et al. 2007). This may result in strong interspecific competition and reproductive isolation pressures (Lanier and Wood 1975; Wood 1982). The latest effort and progress on identification of the aggregation pheromone systems of *I. shangrila* and *P. orientalis* indicates that these two newly described species produced different aggregation pheromone blends from each other and from *I. nitidus* (Zhang et al., in preparation). Such a disparity in pheromone systems (discrimination among their pheromone blends) among the sympatric (competitive or cooperative) bark beetle species and their potential semiochemical interactions may play an important role in maintaining their mass attack sequences (e.g., partial niche separation) and reproductive isolation, and regulating spatial and temporal competition (Birch and Wood 1975; Lanier and Wood 1975; Byers and Wood 1980; Cane et al. 1990; Švihra et al. 1980; Paine et al. 1981; Wood 1982; Byers 1989a, b; Schlyter et al. 1992; Zhang et al. 2008).

Our results suggest that the three major components, 2-methyl-3-buten-2-ol, 74%-(−)-ipsdienol, and (−)-*cis*-verbenol (at 7:2:1), produced by unpaired fed males, are likely the aggregation pheromone components of *I. nitidus*. Another semiochemical found in male hindgut tissue, (−)-*trans*-verbenol, may not be part of the aggregation pheromone system, but it merits further field bioassays to determine its potential functionality. This is the first characterization of an aggregation pheromone system of a bark beetle that is native solely to China. More field-trapping experiments on optimal component ratios (including the enantiomeric ratios of ipsdienol), release rates, and dispenser technology are underway. Traps baited with synthetic aggregation pheromone lures will have great potential as a monitoring and mass-trapping tool in integrated pest management directed against this serious forest pest (Schlyter et al. 2001).

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Variability in Pheromone Communication Among Different Haplotype Populations of *Busseola fusca*

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Abstract The relationship between pheromone composition and mitochondrial haplotype clades was investigated by coupling DNA analyses with pheromone identification and male mate searching behavior among different geographic populations of *Busseola fusca*. The within-population variations in pheromone blend were as great as those observed between geographic populations, suggesting that the female sex pheromone blend was not the basis of reproductive isolation between the geographic clades. Furthermore, while data from wind tunnel experiments demonstrated that most of the tested males were sensitive to small variations in pheromone mixture, there was considerable within-population variability in the observed response. The study identified a new pheromone component, (*Z*)-11-hexadecen-1-yl acetate, which when added to the currently used three-component synthetic blend resulted in significantly higher traps catches. The new recommended blend for monitoring flight phenology and for timing control measures for optimal efficacy of *B. fusca* is (*Z*)-11-tetradecen-1-yl acetate (62%), (*E*)-11-tetradecen-1-yl acetate (15%), (*Z*)-9-tetradecen-1-yl acetate (13%), and (*Z*)-11-hexadecen-1-yl acetate (10%).

Keywords Lepidoptera · Noctuidae · SPME · GC-MS · Synthetic pheromone · Wind tunnel · Field trapping · Reproductive isolation · Clade

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Introduction

The maize stemborer, *Busseola fusca* (Fuller, 1901) is a major pest of *Sorghum bicolor* Moench and *Zea mays* L. in sub-Saharan Africa (Kfir et al. 2002) and now is found rarely on wild host plants (Le Rü et al. 2006; Ong'amo et al. 2006). Due to the internal feeding habit of larvae, insecticide treatments are ineffective unless applied before the young larvae penetrate into the stem (Ndemah and Schulthess 2002). However, sex-pheromone-baited traps could be used to monitor adult flight phenology of *B. fusca* and to determine the timing of pesticide sprays for optimal efficacy. Furthermore, pheromone-based management strategies such as mating disruption or attract- and- kill technology could be developed for use. The sex pheromone of *B. fusca* females was identified by Nesbitt et al. (1980) as a mixture of (*Z*)-11-tetradecen-1-yl acetate (Z11-14:Ac), (*E*)-11-tetradecen-1-yl acetate (E11-14:Ac), and (*Z*)-9-tetradecen-1-yl acetate (Z9-14:Ac), at a ratio of 10:2:2. However, in Kenya and other African countries, this attractant was abandoned as a pest management tool because of poor efficacy.

Recently, Sezonlin et al. (2006) identified three clades of mitochondrial haplotypes of *B. fusca:* W collected from West Africa (Mali, Benin, Ghana, and Togo), and two distinct clades from East Africa: clade I minor and clade II major. Most of the moths collected in Kenya belonged to

clade II. The divergence of these clades occurred around one and a half million years ago (Sezonlin et al. 2006). In this study, we examined the pheromone composition and male response windows both within and between mitochondrial clades, as these parameters are elements of the Specific Mate Recognition Systems (SMRS) sensu Paterson (1985), which may act as strong forces in reproductive isolation in other Lepidoptera species (Frérot and Foster 1991; Pelozuelo et al. 2004).

Methods and Materials

Insects Male and female *B. fusca* were obtained either from the mass rearing unit of ICIPE in Nairobi (Kenya) or from field collected larvae in various localities of Kenya (East Africa) and several countries of West Africa (Table 1). Larvae were fed on artificial diet and, upon pupation, were sent to INRA in Versailles (France), where they were sexed and held in individual containers on moist vermiculite. Adults were collected daily, and males were housed in groups of ten in rectangular crystal polystyrene boxes, while females were placed in individual containers for testing at 2–3 days old. All materials were held at 25°C, 85± 10% rh, under a 12:12 h light/dark reversed photoperiod, and the experiments were carried out during the mating period, which starts in the fifth hour of the scotophase under these conditions (Calatayud et al. 2007).

Chemical Analyses The pheromone glands of calling virgin females were extruded with a thin forceps, and the gland tegument area was gently rubbed for 5 min with the adsorbent part of a 65 µm of Carbowax™-Divinylbenzene (CW/DBV) (SUPELCO) solid-phase microextraction (SPME) fiber (Frérot et al. 1997). The SPME collection of each female was analyzed by gas chromatography (GC) using a Varian 3400 CX with a split-spitless injector heated at 240°C and a FID detector at 260°C. The fiber was subjected to thermal desorption in the injector. A 30 m× 0.32 mm i.d. Rtx®-Wax column (Restek®, France) was used with the oven temperature being ramped from 60°C to 100°C at 15°C/min, then to 245°C at 5°C/min. Helium was the carrier gas (15 psi). GC-MS analyses were carried out on a Varian ion trap Saturn II coupled to a 3400 CX GC. The MS conditions were as following: electron impact (IE) mode, 70 eV, 40 to 330 amu. The GC injector split-splitless was held at 250°C, and the RTX-5 Sil-MS (Restek®, France), 30 m×0.32 mm i.d. column temperature ramped from 50°C to 300°C at 8°C/min. Pheromone compounds were identified by comparing retention times and mass spectra of SPME samples collected from the gland with synthetic reference compounds. We examined, either by GC or GC-MS, 24 females from the ICIPE rearing unit, 21 wild females from various localities in Kenya, and 28 from several West African countries.

Table 1 The quantity (µg) of different synthetic compounds in different *Busseola fusca* pheromone lures (A, B, C, D, E, F) used in wind tunnel experiments

	Female	A	B	C	D	E	F
Z11-14:Ac	80	80	80	80	80	80	75
E11-14:Ac	22	20	20	20	10	10	20
Z9-14:Ac	12	10	5	5	10	20	5
Z11-16:Ac	12	10	10	5	10	10	10

The natural pheromone blend was determined using GC-MS

Wind Tunnel Bioassays The experiments were carried out in a half-cylinder (190 cm long × 80 cm wide × 45 cm high) wind tunnel with an airflow set at 0.6 m/s at 23±2°C and approximately 45% rh. A constant red incandescent light source above the tunnel allowed observations and video recording.

Naive males, placed in individual capped wire mesh cages (3 cm diam × 6 cm), were released from a 12-cm-high platform, 150 cm downwind of the source. Each assay lasted 5 min or ended earlier if the male contacted the pheromone source. Flight behaviors were recorded on video (Panasonic AG-7330) with a Hitachi KP 161 CCD black and white camera equipped with a Nikon objective AF Micro Nikkor 60 mm 1:2.8 D. Tapes were transcribed onto computerized video files and analyzed with The Observer 5.0 software (Noldus, Wageningen, The Netherlands, 2004). The attractiveness of the sources was assessed by using the following parameters (1) latency time, which is the time elapsed from the moment a male was placed on the platform to take-off (LT); (2) upwind flight in the first half (FH); (3) flight to the half-way point (HW); or (4) flight in the second half (SH) of the wind tunnel; and (5) an approach (few millimeters; SA) or either contact or attempt to copulate with the source (C). Males that did not respond were excluded only if their antennae and wings were in bad condition; otherwise, they were classified as not responding.

Stimuli The pheromone glands of two virgin calling females immobilized by a pressure on wing muscles were kept extruded by using crocodile clips, and the insects were placed on a 15-cm-high metal support in the wind tunnel. For any given pair of females, the responsiveness of five males was tested. Synthetic lures for the wind tunnel (Table 1) were prepared with synthetic chemicals applied onto rubber septa (Sigma-Aldrich). All chemicals used were 100% pure for Z9-14:Ac and Z11-16:Ac and about 97% for Z11- and E11-14:Ac.

Field Trapping Field trapping was carried out at Maimahiu (Rift Valley, Kenya). The lures (Table 2), at final concentrations of 1-000 µg loaded onto rubber septa (Sigma-Aldrich), were placed inside multicolored Unitrap traps (Biosystèmes France, SARL; green cover, yellow top, and white bottom) at the edge of maize fields, approximately 1.5 m above the ground and at least 30 m apart. Traps were checked every 2 days, and the lures were renewed every 2 weeks. Each treatment was replicated three times, and traps were checked for five consecutive weeks (N=15) from May 1 to June 20 in 2004.

Genetic Analyses All females used for pheromone identification and males used for wind tunnel bioassays were preserved subsequently in absolute alcohol immediately after the experiment, and total genomic DNA was extracted from their thoraxes and legs by using a DNeasy Tissue Kit (QIAGEN®). A 965-bp fragment of the mitochondrial gene encoding cytochrome *b* was amplified with primers CP1 (5′GAT GAT GAA ATT TTG GAT C 3′) and Tser (5′TAT TTC TTT ATT ATG TTT TCA AAA C 3′). Polymerase chain reactions were performed using a Biometra® thermocycler: initial denaturation for 5 min at 94°C; 40 cycles of 94°C for 1 min, 46°C for 1 min 30 s, and 72°C for 1 min 30 s; and 5 min final extension at 72°C. The reaction mixture contained 5 µl of 5× buffer, 3 µl $MgCl_2$ (25 mM), 1 µl primers (10 mM), 0.6 µl dNTPs (10 mM), 1 µl PCR DNA, and 1 U of Promega *Taq* polymerase per 25 µl of reaction mixture.

For females and males, East and West mitochondrial clades were distinguished by PCR-RFLP using *Bsu*36I restriction digests of the amplified DNA fragment (restriction site: CC-TTAGG), while separation of clades I and II clades was based on *Xho*II restriction digests of the amplified DNA fragment (restriction site: A-GATCC). Reaction mixtures were incubated for 6 h at 37°C, and restriction fragments were separated by agarose gel electrophoresis.

Statistics Data on pheromone identification were analyzed using GLM, while those relating to male behavior data were compared with Student's t test on R 2.4.0 software (Chambers et al. 2006). The mean numbers of moths caught in each field treatment were compared by using Kruskal–Wallis test. For field trapping, the ranks were generated with proc Rank of SAS 9.1 (SAS Institute 2003), analyzed with proc General Linear Model, and then separated by using Tukey–Kramer comparison procedure. For all tests, the significance level was set at $P \leq 0.05$. To determine whether the different populations could be separated on the basis of the pheromone chemical profile, we compared the relative composition of the three minor pheromone components, E11-14:Ac, Z9-14:Ac, and Z11-16:Ac by using principal component analysis (PCA; Georgin 2002).

Results

Chemical Analyses Four different compounds were found in the ICIPE colony females (N=24) (Fig. 1): three tetradecene acetates (1, 2, 3) (m/z=43 (100%), 55 (45%), 67 (97%), 81 (98%), 194 (M^+-60, 24%)) and one hexadecene acetate (4) (m/z=43 (94%), 55 (60%), 67 (100%), 81 (94%), 222 (M^+-60, 17%)). These were identified as (*Z*)-9-tetradecen-1-yl acetate (Z9-14:Ac, 15% (±6.09)), (*E*)-11-tetradecen-1-yl acetate (E11-14:Ac, 27% (±3.70)), (*Z*)-11-tetradecen-1-yl acetate (Z11-14:Ac, 100%), and (*Z*)-11-hexadecen-1-yl acetate (Z11-16:Ac, 14% (±9.58)), by comparing their retention times and mass spectra with those of synthetic compounds.

GC and GC-MS analyses of females revealed that all populations, irrespective of origin, shared the same four components. The component ratios were not significantly different among populations (P<0.05), which was not surprising given the high intra-population variability (Table 3).

PCA results showed that two minor components, Z9-14:Ac and E11-14:Ac, explain 63.4% and 21.9% of the pheromone variability, respectively, while the third component, Z11-16:Ac, explained only 14.6% (data not

Table 2 Catches of *Busseola fusca* males in traps baited with different pheromone lures in the Maimahiu, Rift Valley, Kenya (2004)

	Mixture 1	Mixture 2	Mixture 3	Mixture 4	Mixture 5
Z9-14:Ac	130	130	130	/	13
E11-14:Ac	150	150	/	150	15
Z11-14:Ac	620	620	620	620	62
Z11-16:Ac	100	/	/	/	/
Total male captures	122	85	2	33	66
Mean[a] ± SE male caught per trap/week	8.1±0.4 d	5.7±0.6 c	0.1±0.09 a	2.2±0.5 b	4.4±0.6 b

[a] Means followed by different letters are significantly different from each other according to Tukey–Kramer test at the 5% level

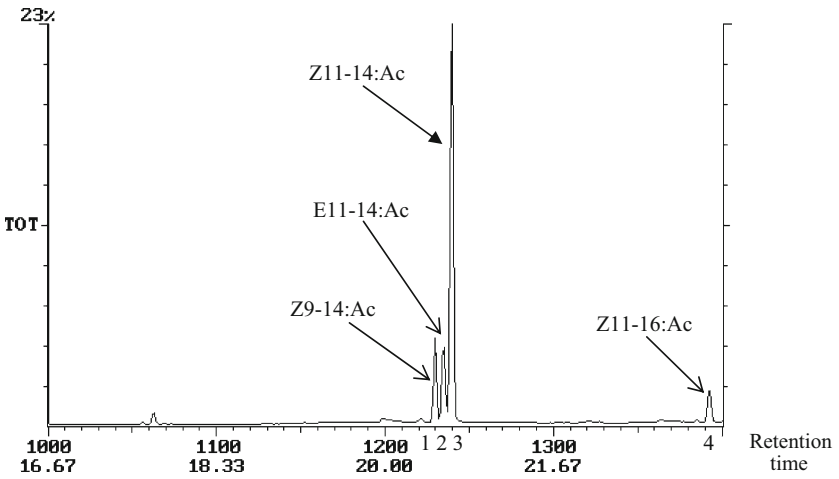

Fig. 1 GC-MS analysis of SPME collection from a single *B. fusca* pheromone gland (RTX-5 Sil-MS, 30 m × 0.32 i. d., IE, 70 eV, 40 to 330 amu)

represented). The PCA analyses did not clearly separate either geographical or mitochondrial populations (Fig. 2).

Wind Tunnel Bioassays The flight behavior of *B. fusca* males to conspecific females (N=20) was similar to that described for most Lepidoptera (Baker 1990; Baker and Haynes 1996). After a highly variable period of latency (mean LT = 51 s; range 10 to 116 s), 85% of the males took flight and exhibited locking-on behavior, with 80% subsequently flying upwind in a low-amplitude zigzag pattern (mean flight time = 69 s; range 10 to 186 s) and touching the female and/or attempting to copulate.

The synthetic lure A, with the same composition as the naturally observed pheromone, was not as attractive as the exposed glands of virgin females, and even minor modifications in the ratio of the minor components reduced upwind flight and/or contact with the source (Fig. 3). The removal of any of the minor components from the identified pheromone mixture abolished the upwind flight response,

Table 3 Differences in the ratios of pheromone components found in the pheromone glands of *Busseola fusca* females from different geographic locations in Africa

	Latitude	Longitude	*N*	Z11-14:Ac (%)	Z9-14:Ac (%)	SD	E11-14:Ac (%)	SD	Z11-16:Ac (%)	SD
East Africa/Kenya										
ICIPE (mass rearing)	S 01°13′13"	E 36°53′46"	24	100	15	6.09	27	3.70	14	9.58
Wild total		20	100	23	12.41	27	8.72	15	12.23	
Eldoret	N 00°34′ 53.28"	E 35°13′ 33.48"	2	100	28	5.99	31	1.51	15	1.07
Kisii	S 00° 46.216′	E 34° 58.788′	1	100	2	/	9	/	2	/
Kisumu	N 00° 35.775′	E 34° 27.165′	2	100	20	9.83	30	0.72	5	4.93
Kitale	N 01° 15.675′	E 35° 10.214′	3	100	27	20.51	23	10.07	26	18.63
Narok	S 01°05′ 32.34"	E 36°07′ 01.62"	4	100	34	13	29	4.14	21	8.12
Rift Valley	S 00°57′ 26.22"	E 36°33′ 28.44"	8	100	18	8.41	27	11.17	13	13.25
West Africa										
Wild total		28	100	25	12.97	23	6.60	18	15.14	
Togo	N 06°10′	E 01°21′	5	100	35	19.47	28	4.72	19	20.78
Burkina Faso	N 12°20′	O 01°40′	2	100	20	13.31	22	21.67	20	21.58
Benin	N 06°30′	E 02°47′	21	100	23	10.46	22	4.89	17	14.09

Ratios are calculated considering the major component, Z11-14 Ac, being 100%

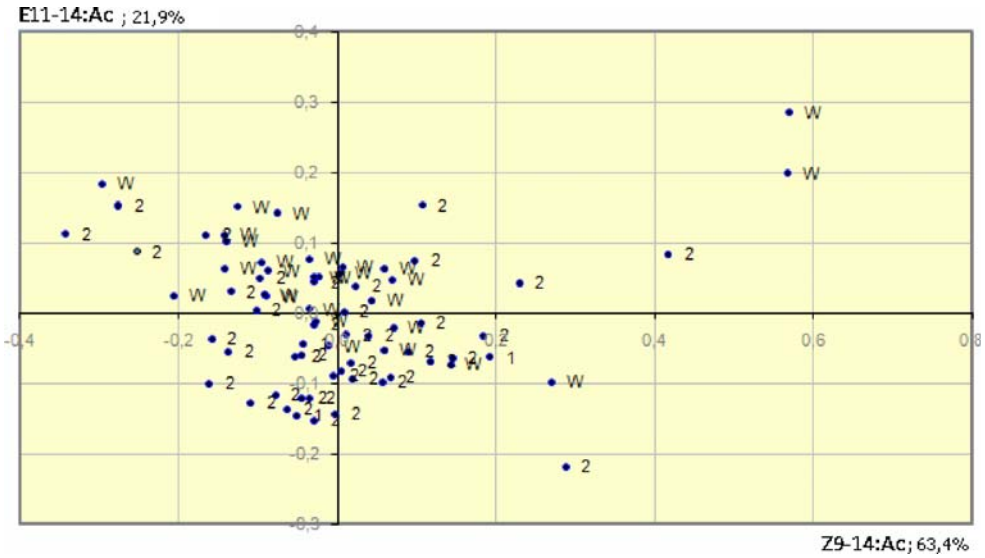

Fig. 2 PCA with two of the four components of *B. fusca* female pheromone: E11-14:Ac and Z9-14:Ac. The three mitochondrial clades are represented as *1*, *2*, and *W* for East I, East II, and West clades, respectively

and low variations in ratio of Z9-14:Ac and E11-14:Ac affected the male responses.

Field Trapping At a loading of 1,000 µg, the addition of Z11-16:Ac (10%) to the previously described three-component blend resulted in significantly higher numbers of males being caught (H=47.9 P<0.001; Table 2). The exclusion in the blend of either Z9-14:Ac or the E11-14:Ac significantly lowers the number of male captures (Table 2).

Genetic Analyses The molecular analyses of 72 *B. fusca* females used for the pheromone identification confirmed the existence of the three, previously described mitochondrial clades (Sezonlin et al. 2006). All females collected in West Africa (N=28) belonged to clade W, while three and 41 East African females fell into clades I and II, respectively. Females from all clades shared the same pheromone components, and the inter-population differences in ratios were not significantly different from intra-population ones (Table 3).

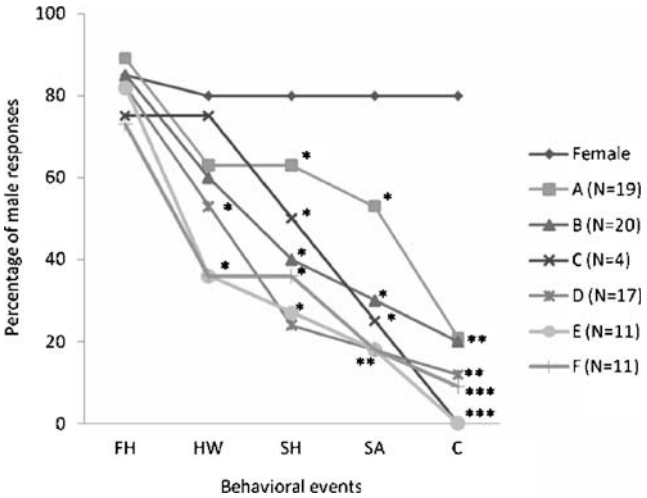

Fig. 3 The response of *B. fusca* males in wind tunnel experiments to the conspecific female sex pheromone and synthetic pheromone mixtures *A*, *B*, *C*, *D*, *E*, and *F*: upwind flight in the first half of the wind tunnel (*FH*); flight to the half-way point (*HW*); flight in the second half of the tunnel (*SH*); source approach (*SA*); contact with the pheromone source or copulation attempts (*C*). Data followed by *asterisk* are significantly different from each other according to Student's *t* test (P<0.05)

Discussion

The addition of the newly identified sex pheromone component, Z11-16:Ac, to the three component blend (Nesbitt et al. 1980) significantly increased *B. fusca* male responses in the wind tunnel. However, despite this improvement, the four-component lure was still less attractive in wind tunnel than the natural female pheromone, even though no other potentially important trace compounds were detected by GC-MS, using single ion monitoring mode. This suggests that the observed behavioral differences can probably be related to wind tunnel conditions that differ from field conditions in Africa and/or to release rate or concentration differences between the septa and female glands. However, the four-component blend of (*Z*)-11-tetradecen-1-yl acetate (62%), (*E*)-11-tetradecen-1-yl acetate (15%), (*Z*)-9-tetradecen-1-yl acetate (13%), and (*Z*)-11-hexadecen-1-yl acetate (10%) was the most effective in the field and offers an improved lure for monitoring *B. fusca* flight activity in IPM programs.

There were no significant interclade pheromone differences, even for clades I and II, which occur in sympatry. Furthermore, while the wind tunnel results showed that even if most of *B. fusca* males were sensitive to small variations of component ratios, the range of male response

windows was high within any given population. Such a plasticity of the male response may explain why the polymorphism of pheromone mixtures does not appear to play a role in reproductive success.

According to Sezonlin et al. 2006, East (clade I and II) and West (W clade) African *B. fusca* populations have been geographically separated for about 1.5 million years. However, we could not relate any particular pheromone blend to a particular mitochondrial clade, suggesting that the female sex pheromone composition did not influence the divergence seen in DNA studies. The sex pheromone mixture and the male responses did not play a role in either reproductive isolation between the sympatric clades, nor for divergence of the SMRS between the now allopatric East and West populations.

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Visualizing a Plant Defense and Insect Counterploy: Alkaloid Distribution in *Lobelia* Leaves Trenched by a Plusiine Caterpillar

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Abstract Insects that feed on plants protected by latex canals often sever leaf veins or cut trenches across leaves before feeding distal to the cuts. The insects thereby depressurize the canals and reduce latex exudation at their prospective feeding site. How the cuts affect the distribution and concentration of latex chemicals was not known. We modified a microwave-assisted extraction technique to analyze the spatial distribution of alkaloids in leaves of *Lobelia cardinalis* (Campanulaceae) that have been trenched by a plusiine caterpillar, *Enigmogramma basigera* (Lepidoptera: Noctuidae). We produced sharp two dimensional maps of alkaloid distribution by microwaving leaves to transfer alkaloids to TLC plates that were then sprayed with Dragendorff's reagent to visualize the alkaloids. The leaf prints were photographed and analyzed with image processing software for quantifying alkaloid levels. A comparison of control and trenched leaves documented that trenching reduces alkaloid levels by approximately 50% both distal and proximal to the trench. The trench becomes greatly enriched in alkaloids due to latex draining from surrounding areas. Measurements of exudation from trenched leaves demonstrate that latex pressures are rapidly restored proximal, but not distal to the trench. Thus, the trench serves not only to drain latex with alkaloids from the caterpillar's prospective feeding site, but also to isolate this section, thereby preventing an influx of latex from an extensive area that likely extends beyond the leaf. Microwave-assisted extraction of leaves has potential for diverse applications that include visualizing the impact of pathogens, leaf miners, sap-sucking insects, and other herbivores on the distribution and abundance of alkaloids and other important defensive compounds.

Keywords Plant-insect interactions · Leaf-trenching · Laticifer · Lobeline · Microwave-assisted extraction · *Lobelia cardinalis* · *Enigmogramma basigera*

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Introduction

When damaged, many plants release defensive exudates such as latices, resins, oils, or mucilages from elongate canal systems. Latex is especially common, occurring in over 20,000 species of plants in 40 families (Lewinsohn 1991), approximately 8% of known plant species. Latex canals are formed of living cells called laticifers that ramify throughout the plant, usually associated with the vascular bundles (Esau 1965; Fahn 1979; Hagel et al. 2008). Latex typically is stored under pressure (Buttery and Boatman 1976); plant injury results in the flow of canal contents down a pressure gradient to the site of damage. Insect herbivores attempting to feed upon latex-defended plants encounter an immediate outflow delivered directly to mouthpart receptors. Latex exudates typically are rich in deterrents and toxins (Farrell et al. 1991; Dussourd 2003; Konno et al. 2004, 2006; Hagel et al. 2008; Sethi et al. 2009); they also congeal rapidly, threatening prospective herbivores with entrapment (Zalucki and Brower 1992; Dussourd 1993, 1995).

Although the double-edged sword of poisons and adhesives delivered by bulk flow is unquestionably an

effective defense against most herbivores, several lineages of insects have evolved behavioral adaptations that circumvent defensive canals (Dussourd 2009 and references cited). The insects often sever leaf veins or cut trenches prior to feeding distal to the cuts (Dillon et al. 1983; Compton 1987; Dussourd and Denno 1991; Dussourd 1999; Evans et al. 2000; Darling 2007). In each case, the vein cuts or trenches reduce exudation beyond the cuts where the insect feeds. Whereas the effects of canal cuts on latex outflow have been well established, little is known about how these injuries alter the distribution and concentration of chemicals in leaves. We used a microwave-assisted extraction technique to visualize and quantify the distribution of alkaloids in leaves of cardinal flower, *Lobelia cardinalis* (Campanulaceae), that have been trenched by a plusiine caterpillar *Enigmogramma basigera* (Lepidoptera: Noctuidae) (Fig. 1). Alkaloids within the latex matrix serve as a tracer that allows us to elucidate how trenching affects the laticifer system and the distribution of latex constituents in the leaf.

Like other plants in the Campanulaceae, *Lobelia* has anastomosing articulated laticifers composed of stacked cells in which the interconnecting walls are partially or completely absent; the resulting tubes are interconnected by cross channels that form a web-like anastomosing structure (Esau 1965; Metcalfe and Chalk 1983). The laticifers in *Lobelia* occur mainly within the phloem tissue, but branches extend into the mesophyll of the leaves (Fraser 1931).

Lobelia cardinalis contains the piperidine alkaloid lobeline at high levels (7.1 mg lobeline/g dry weight, Krochmal et al. 1972) that are comparable to amounts in the closely related medicinal plant *L. inflata* (Indian tobacco). Lobeline acts on nicotinic acetylcholine receptors (Felpin and Lebreton 2004). Overdoses in humans cause stupor, muscle tremors, paralysis, convulsions, coma, and eventually death (Duke 1985). Despite risks of poisoning, *L. inflata* has been used for centuries as a respiratory stimulant and for other medical applications; lobeline currently is being studied as a potential treatment for methamphetamine addiction, dementia and other CNS disorders (Felpin and Lebreton 2004). In addition to lobeline, cardinal flower contains at least three other alkaloids (Chaubal et al. 1962; Krochmal et al. 1972); lobinaline is reportedly the most abundant alkaloid in the plant (Gupta and Spenser 1971).

Fig. 1 Penultimate instar *Enigmogramma basigera* feeding on the tip of a *Lobelia cardinalis* leaf after transecting the leaf with a trench

Cardinal flower in central Arkansas often is heavily damaged by the plusiine caterpillar *Enigmogramma basigera* (Walker), the pink-washed looper (Fig. 1). *Enigmogramma basigera* is classified in the tribe Argyrogrammatini (LaFontaine and Poole 1991) together with other known trenchers such as *Trichoplusia ni* (cabbage looper) and *Pseudoplusia includens* (soybean looper) (Dussourd and Denno 1994). Curiously, late instar *E. basigera* cut trenches while facing the leaf petiole, unlike other trenching plusiines that usually trench facing the leaf tip.

Using *L. cardinalis* and the caterpillar *E. basigera* as a model system, we tested if trenching reduces alkaloid levels at the feeding site distal to the trench. Our approach was to extract alkaloids from *L. cardinalis* leaves by using an adaptation of the protocol developed by Nitao and Zangerl (2004). They used microwaves to extract furanocoumarins from parsnip, *Pastinaca sativa* (Apiaceae), onto phase separation paper to create two dimensional maps of furanocoumarin distribution in entire leaves. Furanocoumarins in parsnip are sequestered primarily within oil ducts found within the leaf veins. Chambers et al. (2007) subsequently published a microwave print of a parsnip leaf trenched by the cabbage looper, *Trichoplusia ni*. The print shows a substantial accumulation of furanocoumarins along the trench, presumably due to drainage of the oil ducts. We significantly modified the microwave technique to extract alkaloids and developed a method for quantifying the distribution of alkaloids in trenched and control (non-trenched) leaves.

Methods and Materials

Study Organisms Lobelia cardinalis L. plants were started from seed (Easyliving wildflower, Willow Springs, MO, USA) and grown in environmental chambers (Model E15, Conviron, Ottawa, Canada) under high pressure sodium and metal halide lamps. Plants were provided with a 12 h day length and a maximum light intensity of 700 µmol/m^2/sec for 4 h; light intensities on either side of the daily maximum

were ramped up/down to simulate natural conditions. Environmental chamber temperatures ranged from 15ºC at night to a daytime high temperature of 25ºC. Plants were fertilized bi-monthly with a 100 ppm nitrogen solution (Scott, Maryville, OH, USA). All experiments used ~9-month-old plants in the rosette stage.

A lab colony of *E. basigera* was established with larvae collected on *L. cardinalis* and adults attracted to mercury vapor lamps in Conway, Arkansas. For experiments, caterpillars were reared from egg hatch until the mid-penultimate instar on potted *L. cardinalis* at low density, thus ensuring that all caterpillars used in the bioassays were experienced trenchers. Trenched leaves were obtained by caging larvae individually on leaves, allowing them to complete a trench that transected the leaf blade, then removing each larva at the first sign of feeding. In all experiments, caterpillars were used only once.

Microwave-assisted Extraction Protocol Twenty *L. cardinalis* plants were randomly assigned to the control or trenched treatment. To maximize efficiency of microwave extraction, only large, flat leaves (> 7.5 cm length, >1.8 cm wide) were selected. For the trenched treatment, caterpillars were allowed to complete a trench (one trench/plant), then the leaf was removed immediately. Ten control and ten trenched leaves were harvested by cutting and cauterizing the petiole with a red-hot scalpel; as documented in a subsequent experiment, this step minimized latex loss from ruptured laticifers.

Alkaloid visualization and analysis involved four steps: 1) partially drying the leaf, 2) microwave-assisted transfer of alkaloids from leaf to TLC plate, 3) developing color by spraying the TLC plates with Dragendorff's reagent, and 4) digital imaging and processing. The excised leaf was first dried at 50ºC for 45 min to reduce water content and thus facilitate alkaloid extraction. The leaf then was sandwiched between two acetate-backed thin layer chromatography (TLC) plates (20×20 cm Whatman silica gel-G cut into 10×10 cm squares, 250 µm thick, no indicator, Fisher Scientific, Pittsburgh, PA, USA). The TLC squares were saturated with toluene, which greatly increased the efficiency of alkaloid extraction from the partially dried leaves. We conducted extensive testing of alternate media for the microwave extraction, including KC-18 reverse phase preparatory TLC plates, alumina oxide preparatory TLC plates, 1PS phase separation paper, SG-81 cation exchange paper, DE-81 anion exchange paper, and P-81 silica impregnated paper (all Whatman media; Fisher Scientific). The different media were tested in combination with solvents (hexane, methylene chloride, chloroform, ethyl acetate, isopropanol, ethanol, methanol, and water). Silica-gel G TLC plates with toluene and cation exchange paper used with acidified solvents both provided high quality leaf prints; we opted for the TLC plates because of their superior resolution.

The *L. cardinalis* leaf pressed between two TLC plates was placed between two 20×20 cm cardboard blotters and two 5 mm thick, 20×20 cm Pyrex glass plates. The leaf assembly was transferred to a 1200 watt microwave oven (Panasonic Model NN-H504BF); a 4.4 kg sand weight in a Pyrex dish was placed on top to compress the assembly and maximize contact between the leaf and TLC plate. The entire assembly was microwave extracted at 30% power for 11 min (the Panasonic magnetron cycles on and off to produce an overall average of 30% power). Long extraction times at lower average power minimized the lateral movement of dissolved alkaloids, which would distort the spatial prints of alkaloid distribution. After microwaving, both the top and bottom TLC plates were sprayed with freshly prepared Dragendorff's reagent (Harborne 1984) yielding a top and bottom print of alkaloid distribution for each leaf extracted (Fig. 2a, b). Leaf prints were allowed to develop for 1 h, then were photographed on a copy stand with a 6 megapixel Minolta Maxxum 7D digital SLR camera equipped with a 70–200 mm zoom lens. To prevent loss of pixel intensity data due to software compression, the images were captured as RAW files, then converted to TIFF format. TIFF images were imported into Adobe Photoshop elements 2.0 where the characteristic yellow background color imparted by Dragendorff's reagent was removed by using a color deconvolution plug-in (Berger et al. 2006). The modified images retained only the orange color that Dragendorff's reagent produces in response to alkaloids (Fig. 2c). This image was converted to eight bit grayscale with black indicating high alkaloid levels, white denoting low levels, and 254 shades of gray representing intermediate levels. The grayscale image was imported into Image J v. 1.37c (National Institute of Health) for quantifying alkaloid levels in the leaf by averaging gray values (Fig. 2d).

To estimate alkaloid content, a standard curve was generated by using eight solutions (10 mg/ml to 0.09 mg/ml) of L-lobeline HCl (Sigma-Aldrich, St. Louis, MO, USA) in methanol. Preliminary testing determined that concentrations in excess of 12 mg/ml resulted in average gray values approaching zero, which effectively set the upper limit of the standard curve. Conversely, lobeline solutions of less than 0.09 mg/ml resulted in average gray values approaching the highest possible value (255), thus setting the lower detection threshold possible by using eight bit digital images. Replicated 30 µl aliquots of the eight solutions were dispensed onto TLC plates identical to the plates used for leaf prints. The plates were sprayed with Dragendorff's reagent, allowed to dry at room temperature for 1 h, then were photographed and processed as previously described with the leaf prints. Image J was used

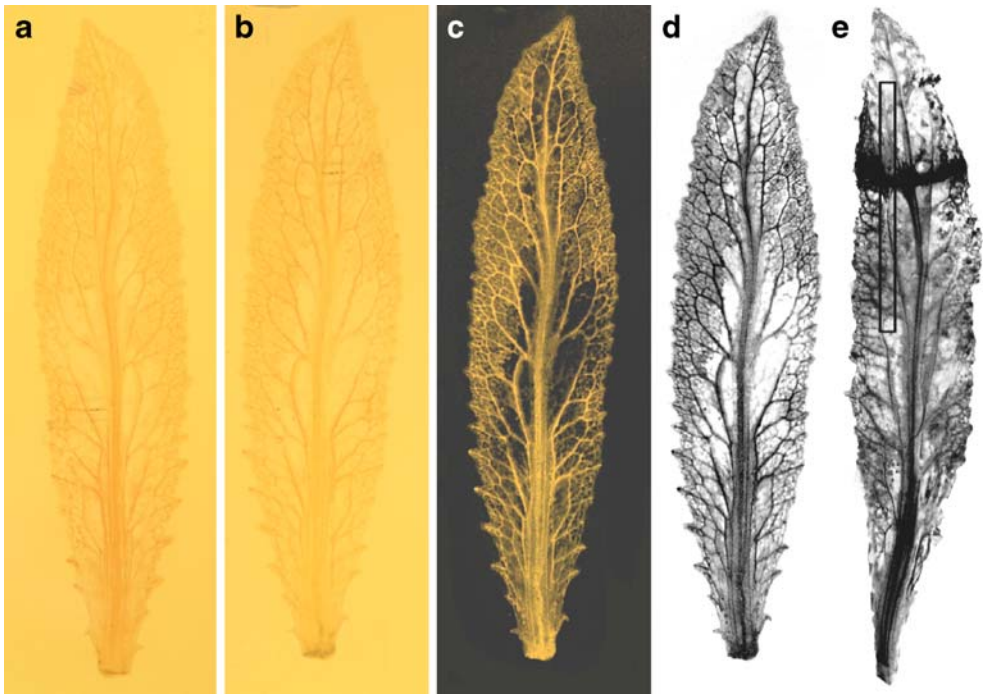

Fig. 2 Alkaloid distribution in *Lobelia cardinalis* leaves as visualized by the microwave extraction procedure. Top (**a**) and bottom (**b**) print of a control leaf. (**c**) The same print as b after removal of the yellow background with color deconvolution. (**d**) Final gray scale image of the same print. (**e**) Gray scale image of a trenched leaf showing alkaloid deposition in the trench. The rectangular sample box is shown to the left of the midrib

to measure the average gray value within a 0.5 cm^2 circle around each 30 µl sample spot. The log_{10} transformed mass of L-lobeline HCl in each spot was regressed against the mean average gray values obtained from image J to produce the regression formula (Log_{10} (Mass of L-Lobeline)=−0.0071(Average gray)+2.5002; R^2=0.96). This formula was used to convert average gray values from leaf prints to alkaloid levels. The calculated alkaloid level was adjusted for area. For example, for a given average gray level, a 0.1 cm^2 sample box was assumed to contain 1/5 of the alkaloid found in the 0.5 cm^2 circle used with the standards.

The quantification of alkaloids in leaf prints is possible because the orange color produced by the Dragendorff reaction increases in intensity as alkaloid titers increase and digital images encode intensity information in numeric form. We spatially calibrated microwave leaf prints by using a metric ruler photographed with each digital image and the scale function of image J. A rectangular sample box measuring 0.25 by 5.0 cm was positioned parallel to the leaf midrib. On trenched leaves, the sample box was situated such that 2 cm of the sample box lay on the distal side of the trench toward the leaf tip, and 3 cm extended proximal to the trench toward the leaf petiole (Fig. 2e). The distance from leaf tip to trench averaged 3.1 cm; therefore, control leaves were sampled by aligning the 2 cm mark of the sample box at 3.1 cm from the leaf tip. Average gray values were sampled along the length of the sample box by using the "plot profile" function of image J, which samples and collects average gray values of pixels every 0.01 cm. We smoothed this raw data by averaging gray values every 0.1 cm. Thus, each 5.0 cm sample box resulted in 50 measurements for every leaf print. For each 0.1 cm slice, the average gray value was converted to mass of lobeline per 0.025 cm^2 (the area of the slice) by using the standard curve. The alkaloid content of corresponding slices from top and bottom leaf prints were added to calculate total alkaloids. Finally, average alkaloid concentrations for both trenched and control leaves were plotted against distance along the sample transect (Fig. 3). We used this graph to separate the leaf into three regions determined by the intersection points of the lines for trenched and control leaves. We designated the section from 0–1.1 cm as the distal (leaf tip) region, from 1.2–2.3 cm as the trench region, and the remaining section from 2.4–4.9 cm as the proximal leaf region.

To test if trenching alters the spatial distribution of alkaloids, we used a 2-way *ANOVA* with fixed effects design. The main effects were treatment (trenched or control) and leaf region (distal, trench, and proximal). The alkaloid content of slices in each leaf region was averaged for each plant resulting in a balanced design with 60 alkaloid measurements (three leaf regions, ten plants/treatment, and two treatments).

One-way *ANOVAs* were used to test the effects of leaf region on alkaloid levels independently for trenched and control leaves. Variances were not homogenous, so we used *Tamhane T-2 post-hoc tests* to compare leaf regions in alkaloid levels. Independent sample *t*-tests were used to compare alkaloid levels across treatments (trenched vs. control) for each leaf region. All statistical analysis was conducted using SPSS v. 11.0. Further details on the

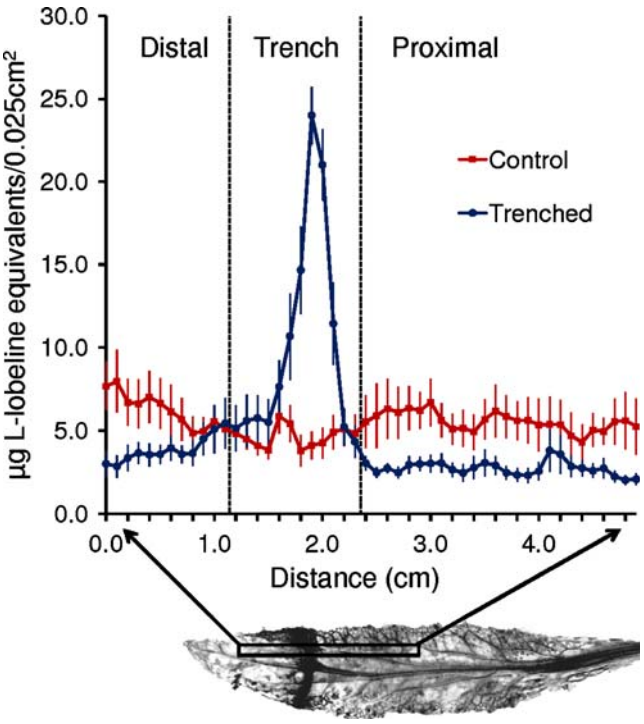

Fig. 3 Distribution of alkaloids in rectangular sample boxes from 10 trenched (blue) and 10 control (red) leaves. The 0.25×5.0 cm sample boxes were divided into fifty 0.25×0.1 cm slices. Each data point represents the average alkaloid content of a slice from ten plants. Alkaloid levels are quantified as lobeline equivalents per 0.025 cm^2 slice; bars represent±one standard error. The trench is clearly denoted by a marked increase in alkaloid levels. Three regions were defined for statistical analysis: a distal region (toward the leaf tip on the left), the trench, and a proximal region (toward the leaf base on the right). These regions were demarcated by the intersections of the control line with the trenched line

microwave extraction protocol and alkaloid quantification can be found in Oppel (2008).

Effect of Petiole Cautery on Latex Loss To determine if cauterization trapped latex within leaf laticifers, we quantified the amount of latex that exuded from *L. cardinalis* petioles. A single mature leaf on 60 plants was randomly assigned to one of three treatments: 1) petiole cut once without cauterization, 2) petiole cauterized, then re-cut without cauterization, or 3) petiole cut twice without cauterization. In the cut once treatment, leaf petioles were severed with a sharp scalpel, and latex exuding from the petiole of the detached leaf was collected on pre-weighed filter paper. Latex on the scalpel blade also was wiped on the filter paper. The petiole of cauterized leaves in treatment 2 was cut and cauterized with a red-hot scalpel to stop latex flow from the severed laticifers. After 2 min, the petiole was cut again ~2 mm above the cauterized cut, this time with a scalpel at room temperature; the exuding latex was collected as described previously. In the cut twice treatment, the leaf petiole was severed with a scalpel at room temperature and allowed to exude latex for 2 min; latex flow ceased well before the 2 min period finished. None of the latex was collected. The petiole then was severed without cautery~2 mm above the initial cut. This time the exuding latex was collected and quantified. If cauterization seals laticifers with no loss of latex, then treatments 1 and 2 should emit similar quantities of latex. If cauterization reduces latex loss, then treatment 2 should release more latex than treatment 3. If cauterization does not seal laticifers due to internal or external bleeding, then treatments 2 and 3 should exude similar quantities of latex. Treatments were compared using a *one-way ANOVA*. Since variances were not homogeneous, *Tanhame's T-2 tests* were used for *post-hoc* comparisons.

Effect of Trenches on Distal Latex Exudation To determine how trenching affects latex exudation beyond the trench where the caterpillars feed, we measured the amount of latex exuding from simulated feeding on trenched leaf tips and paired control leaves. One mature leaf on 10 *L. cardinalis* plants was trenched by a penultimate instar *E. basigera*, whereas another leaf on each plant served as a control. The leaf tip beyond the trench was cut repeatedly in~2 mm slices perpendicular to the leaf midrib, progressing from the leaf tip to the trench. Latex exudate from both sides of each cut was collected onto pre-weighed filter paper; the scissors were wiped on the filter paper between each cut. The same procedure was used with the paired control leaf; slices were removed the same distance from the tip as in the trenched leaf. Leaves were randomly assigned to treatment, and the order of sampling also was randomly determined. The wet weights of latex collected from control and trenched leaves were compared using a paired *t*-test.

Latex Exudation Distal and Proximal to Trench The microwave experiment documented that trenching reduces alkaloid titers on both sides of the trench. Caterpillars feeding on the two sides would presumably ingest comparable amounts of alkaloid unless latex pressures are unequal. To determine if pressures differ, we measured latex exudation from each side. Penultimate instar *E. basigera* larvae were allowed to trench mature *L. cardinalis* leaves (N=10 plants; one caterpillar and one leaf per plant). When the trench was complete, the leaf was cut in~2 mm slices perpendicular to the leaf midrib. After each cut, latex exuding from both sides of the cut was collected and weighed. Sampling progressed either from the leaf tip to the trench (distal) or from the trench toward the leaf petiole (proximal). Whether the distal or proximal regions were sampled first was determined randomly. An identical number of cuts were made on each side of the trench. The

wet weights of latex collected were compared by using a paired sample *t-test*.

Results

Alkaloid Distribution Microwaving leaves provided high resolution two dimensional maps of alkaloid distribution (Fig. 2). Leaf edges were sharp in the prints, indicating that alkaloids did not bleed sideways distorting the prints. The highest concentrations occurred in the leaf veins where laticifers are concentrated in *Lobelia* (Fraser 1931). Alkaloid levels measured for entire top and bottom prints did not differ (t=0.478, df=19, P=0.638), indicating that extraction occurred equally from both leaf surfaces.

The total alkaloid content of trenched leaves (41.8±7.4 μg/cm^2) and control leaves (46.2±6.2, mean±1se) did not differ significantly (t=0.454, df=18, P=0.655). However, trenching profoundly altered the distribution of alkaloids in the sample rectangles. In trenched leaves, alkaloids drained from both distal and proximal leaf regions into the trench. This movement resulted in a significant interaction between treatment (control or trenched) and leaf region (distal, trench, and proximal) in the two-way ANOVA (Fig. 4). Alkaloid content did not differ between leaf regions in the control leaves (one-way ANOVA, $F_{2,\ 27}$=0.599, P=0.557), but did differ in trenched leaves (one-way ANOVA $F_{2,\ 27}$=176.167, P<0.001). In trenched leaves, the distal and proximal leaf regions did not differ in total alkaloids (*Tamhane T-2*, P=0.476); however, each of these regions was lower in alkaloids than the trenched section (*Tamhane T-2*, distal P=0.05, proximal P=0.002)(Fig. 4). In our sample rectangle, alkaloid movement in trenched leaves caused an average loss of 24.02 μg L-lobeline HCl equivalents from the distal region (38% loss), an increase of 53.87 μg L-lobeline HCl equivalents in the trenched region (114% gain), and a decrease of 28.30 μg L-lobeline equivalents in the proximal leaf region (51% loss). The total loss from the proximal and distal regions (52.32 μg) approximately equaled the increase in the trenched region (53.87 μg).

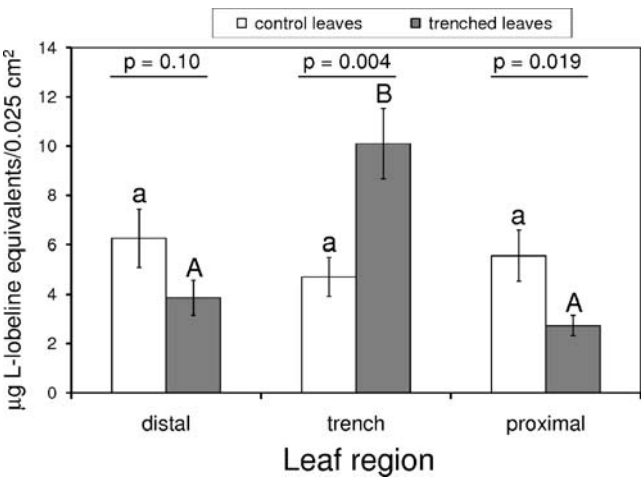

Fig. 4 Alkaloid levels in rectangular sample boxes from 10 trenched and 10 control *Lobelia cardinalis* leaves. Each box was separated into three leaf regions: distal to the trench, trenched section, and proximal to the trench. Alkaloid levels for each region were quantified as average lobeline equivalents per 0.25×0.1 cm slice. Each bar represents the mean±1SE for ten plants. Significant differences between the three leaf regions are denoted by different letters above the bars (lowercase letters for control leaves, uppercase letters for trenched leaves) (one-way ANOVAs; *post-hoc* Tamhane T-2). Significant differences between control and trenched leaves for each leaf region are indicated above the line connecting the contrasting pair (independent sample *t*-tests)

Comparisons between trenched and control leaves revealed that the trenched region was significantly higher in alkaloids than the comparable area in control leaves (t=3.281, df=18, P=0.004), whereas trenching reduced alkaloid levels in the proximal leaf region (t=2.568, df=18, P=0.019) (Fig. 4). The trench drained ~51% of the total alkaloids from the proximal section, suggesting that at least half of the alkaloids in leaves are contained within the latex. Surprisingly, trenched and control leaves did not differ significantly in the alkaloid content of the distal region (t=1.755, df=18, P=0.100). However, alkaloid levels in trenched and control leaves were similar near the trench, but differed substantially farther from the trench (closer to the leaf tip) (Fig. 3).

Alkaloid content in the proximal leaf section of the trenched leaves changed little along the sample box, implying that the area of the leaf drained by the trench extends well beyond our sample box (Fig. 3). Extrapolated linear regression lines for control and trenched leaves in the proximal region intersect at~20 cm from the trench, thus suggesting that trenching drains latex from the entire leaf, perhaps even beyond the petiole. In the distal region of trenched leaves, the low alkaloid levels furthest from the trench likewise imply that latex also has drained from more distant regions.

Effect of Petiole Cautery on Latex Loss Cauterizing petioles reduced, but did not eliminate latex loss from excised leaves. The three treatments differed in the amount of latex emitted from severed petioles (Fig. 5, *one-way ANOVA* $F_{2,57}$=20.48, P<0.001). Leaves cut one time exuded significantly more latex than cauterized leaves (*Tahame T-2*, P=0.007) or leaves cut two times without cautery (*Tahame T-2*, P<0.001). Cauterized leaves exuded more latex than leaves cut two times (*Tahame T-2*, P=0.001). The cauterized leaves released~1.7 times more latex than the leaves cut twice, documenting that cauterization maintained latex pressures within the laticifers. However, the cauterized leaves released substantially less

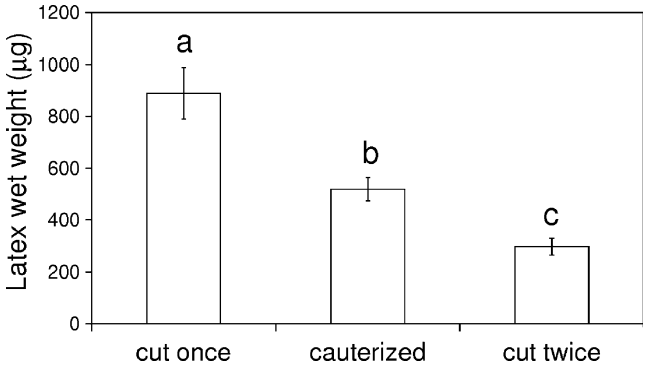

Fig. 5 The amount of latex (mean±1 SE) emitted from *Lobelia* leaf petioles in three treatments: 1) petiole cut once, 2) petiole cauterized, then re-cut, and 3) petiole cut two times (N=20 leaves/treatment). In treatments 2 and 3, latex was collected only from the second cut. Different letters indicate significant differences at the 0.01 level (one-way ANOVA; post-hoc Tamhane T-2)

latex than the control (cut once) leaves, indicating that some latex pressure was lost due to internal or external bleeding (Fig. 5).

Effect of Trenches on Distal Latex Exudation Trenching caused a decrease in the amount of latex that exuded from the leaf tip distal to the trench (paired t=2.757, df=9, P=0.022) (Fig. 6). The trench reduced the wet weight of latex emitted by 92.7%.

Latex Exudation Distal and Proximal to Trench The distal section of the trenched leaf emitted less latex than the proximal region (paired t=3.349, df=9, P=0.009) (Fig. 6). Any caterpillar that tried to feed on the proximal side would encounter ~13 times more latex (wet weight).

Discussion

Our leaf prints document that trenching drains alkaloids from both sides of the trench. As a result, the trench becomes greatly enriched in alkaloids. Although leaves of *L. cardinalis* emit only small amounts of latex (~1–2 µl), the raw latex reacts strongly with Dragendorff's reagent, thus suggesting high alkaloid concentrations. Furthermore, extracts of cardinal flower latex purified by solid-phase extraction and analyzed by using reverse-phase high performance liquid chromatography (HPLC) show a compound with a retention time and maximum absorbance comparable to the (-) lobeline HCl standard (Oppel and Kelley, unpublished data). These observations support the conclusion that *L. cardinalis* sequesters alkaloids within the latex, and that laticifers deliver the alkaloids to the trench. Indeed, during trenching, white latex is clearly observed entering the trench. Alkaloid-producing plants often concentrate alkaloids within laticifers (Wink and Roberts 1998). In the opium poppy, *Papaver somniferum* (opium poppy), for example, morphine levels may exceed 25% of the dry weight of latex exudates (Hartmann 1991).

The low alkaloid levels at the most distant locations from the trench in our sample boxes indicate that the area drained by the trench exceeds the area of our sample box. Previous studies have reported latex drainage from distances of at least 70 cm in severed stems of *Cryptostegia grandiflora* (Apocynaceae) and from a similar distance in tapped rubber trees, *Hevea brasiliensis* (Euphorbiaceae) (Buttery and Boatman 1976). If latex in *Lobelia* likewise flows from distant reserves into leaves during trenching, it is reasonable to expect higher total alkaloid levels in trenched leaves. Yet, there was no significant difference between trenched and control leaves in our study. This apparent contradiction likely can be attributed to an underestimation of alkaloids in the trench. Alkaloid levels above threshold produce the maximum saturation with Dragendorff's reagent and are underestimated by our methods. An alternative explanation for the similar alkaloid levels in trenched and control leaves is that the laticifers in each leaf are isolated as a separate reservoir. However, when *L. cardinalis* petioles are severed, latex drains from both sides of the cut, wherever the petiole is cut, suggesting that laticifers in the stem extend into the leaf.

The reaction of laticifers upon being breached has been studied in *Hevea brasiliensis* and other plants (Buttery and Boatman 1976; Milburn et al. 1990; Pickard 2008). Rupture of the pressurized latex tubes causes latex flow to the injury site down the resulting pressure gradient. Concurrently, water from surrounding tissues flows into the laticifers due to the large difference in water potential. The dilution of latex further facilitates latex flow. The dilution effect can be seen readily in *L. cardinalis* laticifers; the repeated cutting of a stem results in latex exudation that changes from an opaque, viscous, white fluid to a much clearer, watery fluid.

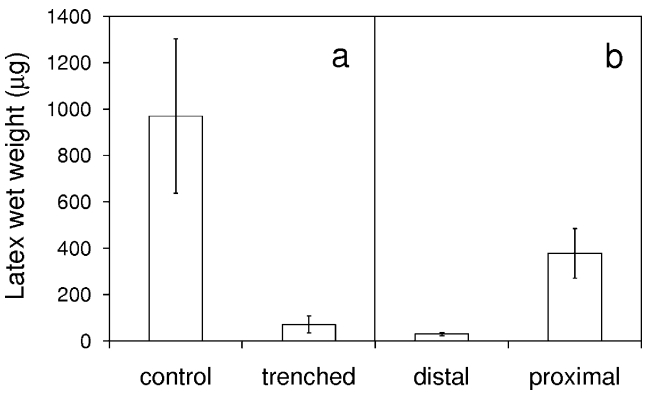

Fig. 6 **a** Amount of latex (mean±1 SE) emitted from (a) paired control and trenched *Lobelia* leaf tips, and **b** distal and proximal to trenches (N=10 leaves/treatment)

Drainage eventually ceases due to plugging (Buttery and Boatman 1976).

In the sample rectangles of our trenched leaves, alkaloid concentrations did not differ distal and proximal to the trench. Evidently, the influx of water into laticifers during drainage diluted the latex similarly on both sides of the trench. Why then do *E. basigera* caterpillars invariably feed on the distal side? Our exudation measurements documented that latex pressures are much lower on the distal side (Fig. 6). Laticifers proximal to the trench are partially recharged quickly because they remain connected to the extensive network of pressurized laticifers that extend throughout the plant. Although trenching drains and dilutes latex from both sides of the trench, only the distal side remains depressurized. The trench thus serves two purposes: it depletes and dilutes latex distal to the trench where the caterpillar feeds, and it prevents the influx of latex to this site from the plant's vast laticifer network. Dussourd and Denno (1991) proposed that trenching functions primarily to block the flow of latex to the feeding site, not to drain latex from the feeding site. Our current analysis documents both blockage and drainage in *L. cardinalis* leaves, illustrates the overwhelming importance of blockage, and shows clearly that latex is not a static defense. Any caterpillar attempting to feed on the proximal side of the trench would experience latex delivered directly to mouthpart receptors from pressurized laticifers. Caterpillars feeding on the distal side can consume the contents of the depressurized laticifers diluted with water and mixed with other leaf chemicals.

The trench drained~51% of the alkaloids from the proximal section of the leaves (Fig. 4), suggesting that at least half of the leaf alkaloids occur in the latex. The remaining alkaloids in trenched leaves must either be residuals not drained from the laticifers or alkaloids sequestered outside of the latex system. That the veins of trenched leaves still reacted strongly to Dragendorff's reagent suggests that most or all of the alkaloids are sequestered within the laticifers, which are closely associated with phloem bundles (Fraser 1931). However, it is conceivable that some alkaloid in *L. cardinalis* is located in the xylem or phloem, and that these tube systems contribute to the delivery of alkaloids to the trench. Alkaloid transport via xylem and phloem have both been documented (Wink and Roberts 1998). The pyrrolidine alkaloid nicotine, a close functional analog of the piperidine alkaloid lobeline, is transported in the xylem of *Nicotiana* (Solanaceae). Conversely, the quinolizidine alkaloids produced by plants in *Lupinus* (Fabaceae) are transported in the phloem, as are the pyrrolizidine alkaloids found in *Senecio* (Asteraceae).

We can readily exclude the xylem as a source of alkaloids moving into the trench in our experiments. The xylem during the day is under negative pressure. Rupture at the trench would cause rapid movement of xylem sap away from the trench as air entered the xylem tubes. Drying the leaf before microwaving should have prevented possible expansion and flow of this sap into the trench during heating in the microwave. In addition, analysis of stomatal conductance beyond *E. basigera* trenches in *L. cardinalis* indicates that many xylem vessels are not severed by the trench (Oppel, unpublished data); thus, bulk flow of xylem sap into the trench during microwaving seems unlikely. Unlike the xylem, the phloem sap is under positive pressure; it could potentially drain into the trench together with latex. Drainage would cease soon due to the formation of phloem protein (p-protein) plugs on the sieve plates (Oparka and Santa Cruz 2000). Nevertheless, we can not exclude a possible contribution of the phloem to the high alkaloid levels noted in the trench in our microwave prints.

Reduced exposure to latex during feeding presumably benefits *E. basigera* larvae. In the closely related cabbage looper, *Trichoplusia ni*, larvae are unable to develop on intact *L. cardinalis* plants, but feed and develop readily on excised leaves that are partially drained of latex (Dussourd 2003). The alkaloid lobeline is strongly deterrent to *T. ni* and triggers trenching behavior (Dussourd 2003). In contrast, *E. basigera* larvae develop and feed readily on intact *L. cardinalis*, although they grow more quickly on excised leaves (Dussourd, unpublished data). Whether lobeline and other piperidine alkaloids in *L. cardinalis* negatively affect *E. basigera* larvae has not yet been tested. Lobeline is known to deter feeding by a ctenuchid caterpillar, *Syntomis mogadorensis*, and by honeybees, *Apis mellifera* (Wink and Schneider 1990; Detzel and Wink 1993).

The microwave extraction protocol has advantages and limitations. The technique is uncomplicated, relatively rapid, and inexpensive when compared to other chemical analysis techniques such as HPLC and GCMS. Additionally, the microwave method yields high resolution, quantitative, two dimensional maps of alkaloid distribution in leaves. Few other techniques provide such detailed information on the distribution of secondary compounds in entire leaves (Nitao and Zangerl 2004). Microwave extraction of alkaloids and quantification should be possible with diverse plant species and diverse secondary compounds where visualization reagents are available. The technique has promise for studying the effects of pathogens, leaf miners, galls, mandibulate and haustellate insects, and various abiotic factors on alkaloid distributions in leaves. The method also might be used for analyzing alkaloid distributions in other flat substrates such as butterfly or moth wings.

Limitations of the microwave method include its inability to quantify very low or high alkaloid concentrations; low levels are undetectable, whereas high levels

are indistinguishable if they exceed the maximum response from Dragendorff's reagent. Intensifiers for Dragendorff's reagent such as sodium nitrite (Rubia and Gomez 1977) might be used to enhance sensitivity for low levels. Shorter microwave times could be used to extract alkaloids at low efficiency for quantifying high concentrations. Since Dragendorff's reagent is known to react with steroids, triterpenes, and some proteins, in addition to alkaloids, false positive responses may occur (Anderson et al. 1977). In *L. cardinalis*, lobeline and other alkaloids are known to occur at high concentrations (Krochmal et al. 1972), and we confirmed that lobeline is indeed found in the latex (Oppel and Kelley, unpublished data). Additional shortcomings of our technique include the inevitable loss of latex from the petiole cut during leaf removal. We explored other methods of minimizing latex loss that include ligating the leaf petiole, which was ineffective in stemming latex loss. Freezing the leaf with liquid nitrogen caused the leaf to crack; alkaloids diffused from the cracks during microwaving. Cautery appears to be the best method. Finally, the extraction efficiency of our microwave procedure remains unknown; therefore, the values reported should be considered as relative amounts, not total alkaloid levels. Nitao and Zangerl (2004) did test the effectiveness of their furanocoumarin protocol and found that microwaving parsnip leaves extracted furanocoumarins with efficiencies varying from 21–67%.

In summary, this study represents to our knowledge the first time that the effect of trenching on the two dimensional distribution of a secondary compound has been quantified. Our technique for visualizing and quantifying alkaloid distribution should have broad application in studies of insect-plant interactions, in monitoring how biotic and abiotic factors affect alkaloid production in economically important plants such as tobacco and poppy, and as a teaching tool accessible to both high school and college students.

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Antitermitic Activities of Abietane-type Diterpenes from *Taxodium distichum* Cones

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Abstract Eight known abietane-type diterpenes were isolated from the weak acidic fraction of the *n*-hexane extract from cones of *Taxodium distichum*, one of the extant, living fossil conifers. They were identified as 6,7-dehydroroyleanone (**1**), taxodal (**2**), taxodione (**3**), salvinolone (**4**), 14-deoxycoleon U (**5**), 5,6-dehydrosugiol (**6**), sandaracopimaric acid (**7**), and xanthoperol (**8**). The structures of these compounds were determined by comparison of NMR spectral data with published data. The antitermitic (termicidal and antifeedant) activities of the compounds **1**–**8** against the subterranean termite, *Reticulitermes speratus* Kolbe, were evaluated. Compounds **1** and **3** showed potent termicidal activity, and **5** and **8** showed potent antifeedant activity. Compound **1** was found to be one of the representative bioactive compounds in the *n*-hexane extract of *T. distichum* cones. Compounds **1**–**8**, with the exception of **7**, were oxides of ferruginol (**9**). Therefore, the presence of various oxidation forms of the abietane-type structure reflects their various bioactivities.

Keywords *Taxodium distichum* · Cone · Taxodiaceae · Abietane · Diterpene · Oxidation · Antitermite · *Reticulitermes speratus* · Bioactivity

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Introduction

Taxodium distichum Rich (Taxodiaceae), commonly known as "bald" or "swamp" cypress, is well known as an extant deciduous, living fossil conifer indigenous to the southern part of North America. *T. distichum* heartwood is used for building materials, and has been reported to resist the attacks of the subterranean termite, *Coptotermes formosanus* Shiraki (Scheffrahn et al. 1988). The cones and seeds of *T. distichum* tend to be discovered from ancient stratum, and there are several reports concerning the fossil conifer and sediments of ancient flora (Otto et al. 2003, 2005). The cones produced by the conifers are essential parts for self-propagation. Consequently, the potential for having anti-feedant, antifungal, as well as other phytochemical activities against external influences is suggested (Yano and Furuno 1994).

Abietane-type diterpenes are widely distributed in the plant kingdom as natural compounds. They reveal characteristic bioactivities including cytotoxic, anti-tumor, anti-microbial, and anti-bacterial effects (Gao and Han 1997; Ulubelen et al. 1999; Gigante et al. 2003; Son et al. 2005; Marques et al. 2006). From previous studies, it is known that the abietane-type diterpenes are the major compounds in the cones of *Taxodium* species (Otto et al. 2003). The isolation of several kinds of abietane-type diterpenes from the cones of *T. distichum* has been reported (Yamamoto et al. 2003). Some of the compounds isolated, such as taxodione, taxodone, and taxodistine A and B, have been reported to have anti-tumor and cytotoxic activity (Kupchan et al. 1969; Hirasawa et al. 2007). Taxodione has been reported to exhibit antibacterial activities against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE) as well (Yang et al. 2001). In addition, nezukol,

ferruginol, and manool, which have been isolated from the *T. distichum* heartwood (Scheffrahn et al. 1988), were shown to have antitermitic activities (Kang et al. 1993; Kano et al. 2004).

Natural compounds isolated from Taxodiaceae extant fossil conifers, such as from the genus *Metasequoia* and *Taxodium*, mostly have been taken from the leaf (Si et al. 2005) or the heartwood (Sato et al. 1966; Enoki et al. 1977). Only a few reports have considered bioactive compounds in living fossil conifer cones. Their bioactive compounds might have been important for the persistence, and thus, evolutionary success of *T. distichum.*

The aim of this research was to investigate the bioactive compounds of *T. distichum* cones and to evaluate their antitermitic activities by using a bioassay with the subterranean termite *Reticulitermes speratus* Kolbe. Subterranean termites are important decomposers in the terrestrial environment, and there has been interest in the activities of natural compounds against these termites (Bultman et al. 1979; Cornelius et al. 1997; Bläske and Hertel 2001). The termites *R. speratus*, *C. formosanus*, and *Cryptotermes domesticus* are harmful insects that damage wooden structures of buildings in Japan (Kang et al. 1993; Fukumoto et al. 2007).

Methods and Materials

Termite Collections Colonies of *Reticulitermes speratus* Kolbe were collected in Tsuruoka city, Yamagata prefecture, and maintained in well-humidified chambers at 27°C± 1°C for 1 month before the bioassay.

Termicidal and Antifeedant Tests Termicidal and antifeedant activities were tested simultaneously. Samples were applied to paper discs (Advantec, 8 mm diam, 1.5 mm thickness, ca. 30 mg disk weight) according to a previously described method (Tellez et al. 2002; Ganapaty et al. 2004). Paper discs were treated with 60 μl of each sample solution (5.0 mg/ml in MeOH). After being dried in a vacuum desiccator for 24 h, the weights of the paper discs were measured. The concentration of the sample in the dried paper disc was prepared to 1.0% (sample weight/paper disc weight×100). This dried paper disc was put on top of sea sand (3 g) that was spread uniformly at the bottom of a glass petri dish (45 mm diam, 20 mm high). Ten *R. speratus* workers were placed in the petri dish. The sea sand was kept moistened with water by using a sprayer. These petri dishes were maintained in a well-humidified chamber at 27°C±1°C for 10 d together with blank paper discs (no samples) and controls (no paper discs). Each of the tests included three replicates. The mortality of termites was recorded every 24 h, and termicidal activities were evaluated from the mortality average. The mass loss of each paper disc was measured at the end of the experimental period. Antifeedant activities were evaluated from the average of the mass that one termite fed per 24 h (mass loss of paper disc in 10 d/total number of termites in 10 d), and the relative rates were calculated from the blank readings.

Plant Material The fallen cones of *T. distichum* (Taxodiaceae) were gathered in the Yamagata Field Science Center (Faculty of Agriculture, Yamagata University, Japan). Identification was confirmed by the above institution.

General Experimental Procedures GLC analysis was performed with a HITACHI G-3000 Gas Chromatograph under the following conditions: DB-1 capillary column (30 m×0.32 mm i.d.; 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA); column temperature from 180°C (0 min) to 280°C (15 min) at 4°C/min; injection temperature 250°C: detection temperature 250°C. GC/MS data were measured with a SHIMADZU QP-5000 GC-MS: DB-1 capillary column (30 m×0.32 mm i.d.; 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA); column temperature from 100°C (1 min) to 320°C (10 min) at 5°C/min; injection temperature of 230°C: detection temperature of 250°C; acquisition mass range of 450-50 amu using helium as the carrier gas (3.6 ml/min). 1D and 2D NMR spectra were measured with a JEOL JNM-EX400 (^{1}H 400 MHz/^{13}C 100 MHz) spectrometer. The IR spectra were taken by a HORIBA FT-710 IR spectrometer with KBr pellets. UV spectra were taken by a SHIMADZU UV-1600PC spectrometer. Optical rotation values were measured by a HORIBA SEPA-300 polarimeter.

Extraction and Isolation The *n*-hexane extract of air-dried *T. distichum* cones (800.0 g) was extracted by the same method as previously reported (Kusumoto et al. 2008). The *n*-hexane soluble fraction (88.6 g) was extracted by partition extraction with saturated $NaHCO_3$, 10% $NaCO_3$, and 1% NaOH aqueous solution in a separatory funnel to yield 1.4 g, 1.2 g, and 11.2 g fractions, respectively, along with 74.8 g residue fraction. Part of the weak acidic fraction (11.0 g) was applied to silica gel 60 N (spherical 63–210 μm, neutral, Kanto Chemical Co., Japan) column chromatography (CC) with solvent systems of *n*-hexane / EtOAc (100:1 to EtOAc only), and eight fractions (fr. A–H) were collected. Fractions C, D, and E (566.3 mg, *n*-hexane / EtOAc, 100:1) were recrystallized by using benzene to give a red needle of 6,7-dehydroroyleanone (**1**, 306.3 mg). Further, fr. G (541.9 mg, *n*-hexane / EtOAc, 100:3) was recrystallized by using *n*-hexane to give a colorless needle of taxodal (**2**, 44.6 mg). Fraction F (304.7 mg, n-hexane /

EtOAc, 100:1 to 50:1) was purified by silica gel 60 N CC with a solvent system of *n*-hexane / chloroform (3:1 to chloroform only), and 40 fractions (fr. 1–40) were collected. Fractions 9, 10, 11, and 12 (110.0 mg, *n*-hexane / chloroform, 3:1) were purified by silica gel 60 N CC using a solvent system of *n*-hexane / chloroform (3:1) to give a dark yellow amorphous solid of taxodione (**3**, 29.0 mg). Fraction H (10.2 g) contained a polymerized mixture, which was separated to yield the EtOAc soluble (9.8 g) and insoluble fractions (333.7 mg). The EtOAc soluble fraction (6 g) was applied to silica gel 60 N CC using chloroform, and 48 fractions (fr. H1-H48) were collected. Fractions H9, H10, and H11 (233.1 mg) were recrystallized by using benzene, which gave a pale yellow powder of salvinolone (**4**, 123.2 mg). Successively, fractions H16, H17, H18, and H19 (461.1 mg) were recrystallized by using benzene, which yielded a pale yellow crystal of 14-deoxycoleone U (**5**, 182.8 mg). Fractions H23 to H27 (921.8 mg) were applied to silica gel 60 N CC using a solvent system of benzene / acetone (9:1) to a give pale yellow needle of 5,6-dehydrosugiol (**6**, 21.3 mg), a dark brown needle of sandaracopimaricacid (**7**, 11.6 mg), and a yellow needle of xanthoperol (**8**, 37.6 mg), respectively. Ferruginol (**9**) and sugiol (**11**) were isolated from *Cryptomeria japonica* in previous studies (Nagahama et al. 2000; Ashitani et al. 2001). 6,7-Dehydroferruginol (**10**) was synthesized from **11** by a chemical conversion according to a previous report (Matsui et al. 2004).

*6,7-Dehydroroyleanone (**1**)* 306.3 mg. Red needle. IR γ_{max} (KBr) cm^{-1}: 3360.4, 2961.2, 2925.5, 2910.1, 2869.6, 1663.3, 1642.1, 1625.7, 1551.5, 1457.9, 1377.9, 1329.7, 1298.8, 1272.8, 1253.5, 1164.8, 1106.0, 913.1, 769.5, 756.0, 714.5, 651.8; UV (C=0.05 mg/ml, *n*-hexane) nm: 451.0, 327.0, 255.5; mp: 171°C; $[\alpha]_D^{24.3}=-364.8°$ (C=0.3 mg/ml); EI-MS: *m/z* 314 (M^+, $C_{20}H_{26}O_3$, 79%), 299 (27), 281 (6), 271 (21), 258 (16), 253 (16), 246 (15), 245 (68), 244 (74), 243 (23), 232 (100), 231 (45), 229 (22), 217 (23), 213 (25), 201 (15), 187 (25), 185 (19), 129 (18), 128 (21), 115 (32), 91 (24), 83 (41), 69 (30), 55 (75); ^{1}H-NMR ($CDCl_3$): δ 0.98 (3H, *s*, 18-CH_3), 1.01 (3H, *s*, 19-CH_3), 1.03 (3H, *s*, 20-CH_3), 1.21 (3H, *d*, *J*=7.1 Hz, 16-CH_3), 1.22 (3H, *d*, *J*=7.2 Hz, 17-CH_3), 1.25 (2H, *m*, 3-CH_2), 1.43 (2H, *td*, *J*=13.3 Hz, 3.9 Hz, 1-CH_2), 1.51 (2H, *m*, 3-CH_2), 1.62 (2H, *m*, 2-CH_2), 1.71 (2H, *m*, 2-CH_2), 2.14 (1H, *t*, *J*=3.1 Hz, 5-CH), 2.89 (2H, *d*, *J*=3.4 Hz, 1-CH_2), 3.17 (1H, *hept*, *J*=7.1 Hz, 15-CH), 6.46 (1H, *dd*, *J*=3.0 Hz, 9.7 Hz, 6-CH), 6.81 (1H, *dd*, *J*=3.1 Hz, 9.8 Hz, 7-CH), 7.34 (1H, *s*, 12-OH); ^{13}C-NMR ($CDCl_3$): δ 15.2 (20-CH_3), 18.7 (2-CH_2), 19.8 (16-CH_3), 20.0 (17-CH_3), 22.8 (19-CH_3), 24.1 (15-CH), 32.6 (18-CH_3), 33.3 (4-C), 35.2 (1-CH_2), 39.3 (10-C), 40.5 (-CH_2), 52.1 (5-CH), 121.1 (7-CH), 122.6 (13-C), 138.5 (8-C), 139.6 (6-CH), 140.5 (9-C), 151.2 (12-C), 183.5 (11-C), 186.1 (14-C). (Figs. 1 and 2).

*Taxodal (**2**, Fig. 1)* 44.6 mg. Colorless needle. IR γ_{max} (KBr) cm^{-1}: 3012.3, 2969.8, 2948.6, 2861.8, 2775.1, 2751.9,

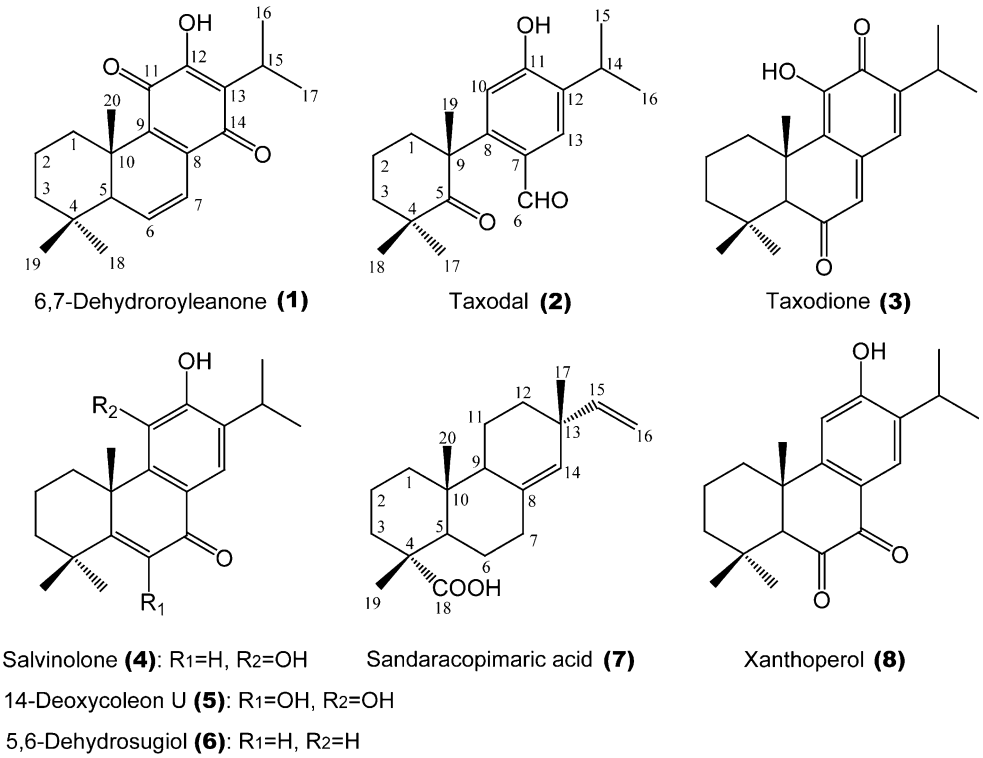

Fig. 1 Structures of compounds isolated from *Taxodium distichum* cones

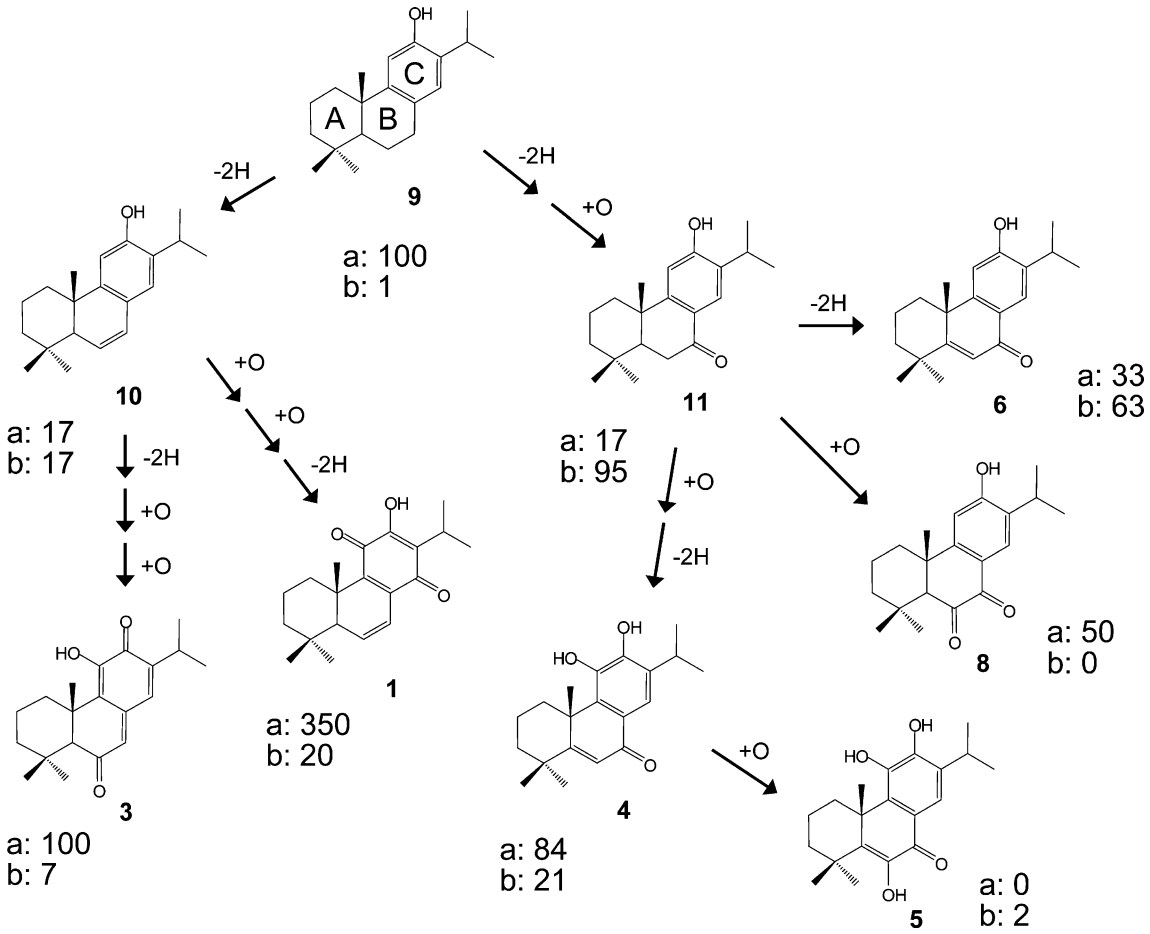

Fig. 2 Route of ferruginol (**9**) oxidation via 6,7-dehydroferruginol (**10**) and sugiol (**11**), and bioactivities of each compound. **a** Relative mortality (%) of Reticulitermes speratus caused by the compounds; mortality caused by compound **9** in 10 d=100 (A higher rate means a higher termicidal activity). **b** Relative mass (μg) of paper disc that one termite fed in 24 h, mass of disc treated with **9**=1 (A lower rate means a higher antifeedant activity)

2503.2, 1689.3, 1666.2, 1614.1, 1581.3, 1469.5, 1461.8, 1425.1, 1400.1, 1346.1, 1276.7, 1253.5, 1211.1, 1172.5, 979.7, 898.7, 763.7; UV (C=0.005 mg/ml, EtOAc) nm: 285.5; mp: 234–240°C; $[\alpha]_D^{20}$=−131.4 ° (C=0.5 mg/ml); EI-MS: *m/z* 302 (M^+, $C_{19}H_{26}O_3$, 26%), 287 (3), 274 (8), 269 (2), 259 (9), 256 (3), 241 (5), 232 (9), 231 (51), 220 (55), 219 (36), 205 (14), 204 (18), 203 (100), 191 (29), 190 (22), 189 (15), 187 (16), 177 (17), 175 (16), 161 (22), 159 (10), 147 (12), 128 (14), 115 (15), 91 (16), 77 (11), 69 (11), 55 (27); ^{1}H-NMR (aceton-d_6): δ 1.17 (3H, *s*, 17-CH_3), 1.22 (3H, *s*, 18-CH_3), 1.26 (3H, *d*, *J*=7.0 Hz, 16-CH_3), 1.27 (3H, *d*, *J*=7.0 Hz, 15-CH_3), 1.55 (3H, *s*, 19-CH_3), 1.62 (2H, *m*, 1-CH_2), 1.67 (2H, *m*, 2-CH_2), 2.43 (2H, *ddd*, *J*=13.3 Hz, 13.3 Hz, 4.0 Hz, 3-CH_2), 3.31 (1H, *hept*, *J*=7.0 Hz, 14-CH), 7.16 (1H, *s*, 10-CH), 7.73 (1H, *s*, 13-CH), 9.20 (1H, *s*, 11-OH), 9.68 (1H, *s*, 6-CH); ^{13}C-NMR ($CDCl_3$): δ 17.6 (2-CH_2), 20.8 (15-CH_3), 20.8 (16-CH_3), 24.0 (19-CH_3), 25.5 (14-CH), 25.6 (17-CH_3), 28.4 (18-CH_3), 36.9 (3-CH_2), 38.9 (1-CH_2), 43.1 (4-C), 51.4 (9-C), 114.3 (10-CH), 124.8 (7-C), 131.4 (12-C), 136.5 (13-CH), 146.9 (8-C), 158.7 (11-C), 190.2 (6-CH), 213.2 (5-C).

*Taxodione (**3**, Figs. 1 and 2)* 29.0 mg. Dark yellow amorphous solid. IR γ_{max} (KBr) cm^{-1}: 3322.8, 2935.1, 2361.4, 1669.1, 1612.2, 1594.84, 1354.8, 639.3; mp: 104-109°C; EI-MS *m/z*: 314 (M^+, $C_{20}H_{26}O_3$, 100%), 299 (17), 286 (58), 272 (19), 271 (83), 253 (13), 245 (69), 245 (69), 244 (25), 243 (25), 232 (38), 231 (44), 229 (25), 217 (33), 215 (26), 206 (22), 203 (25), 189 (22), 187 (22), 175 (14), 173 (15), 165 (12), 157 (11), 141 (17), 129 (20), 128 (24), 115 (30), 109 (41), 91 (26), 77 (24), 69 (31), 55 (51); ^{1}H-NMR ($CDCl_3$): δ 1.12 (3H, *s*, 18-CH_3), 1.16 (3H, *d*, *J*= 7.0 Hz, 17-CH_3), 1.18 (3H, *d*, *J*=7.0 Hz, 16-CH_3), 1.22 (2H, *m*, 3-CH_2), 1.27 (3H, *s*, 19-CH_3), 1.27 (3H, *s*, 20-CH_3), 1.40 (2H, *m*, 3-CH_2), 1.61 (2H, *m*, 2-CH_2), 1.73 (2H, *m*, 1-CH_2), 1.75 (2H, *m*, 2-CH_2), 2.60 (1H, *s*, 5-CH), 2.93 (2H, *m*, 1-CH_2), 3.07 (1H, *hept*, *J*=7.0 Hz, 15-CH), 6.21 (1H, *s*, 7-CH), 6.88 (1H, *s*, 14-CH), 7.58 (1H, *s*, 11-OH);

^{13}C-NMR ($CDCl_3$): δ 18.5 (2-CH_2), 21.2 (16-CH), 21.6 (17-CH_3), 21.8 (20-CH_3), 22.1 (19-CH_3), 27.1 (15-CH), 32.8 (4-C), 33.3 (18-CH_3), 37.0 (1-CH_2), 42.9 (10-C), 63.0 (5-CH), 125.6 (9-C), 134.0 (7-CH), 136.1 (14-CH), 139.9 (8-C), 145.0 (11-C), 145.3 (13-C), 181.7 (12-C), 201.0 (6-C).

*Salvinolone (**4**, Figs. 1 and 2)* 123.2 mg. Pale yellow powder. IR γ_{max} (KBr) cm^{-1}: 3373.9, 3250.4, 2960.2, 2869.6, 2361.4, 2341.2, 1625.7, 1583.3, 1506.1, 1456.0, 1371.1, 1314.3, 1260.3, 1178.3, 1048.1; mp: 204–206°C; EI-MS *m/z*: 314 (M^+, $C_{20}H_{26}O_3$, 99%), 299 (11), 286 (5), 272 (11), 271 (44), 258 (10), 255 (5), 245 (89), 244 (100), 229 (34), 215 (29), 203 (20), 175 (14), 128 (10), 115 (12), 83 (18), 55 (24); ^{1}H-NMR ($CDCl_3$): δ 1.27 (3H, *d*, *J*=7.0 Hz, 16-CH_3), 1.30 (3H, *d*, *J*=7.0 Hz, 17-CH_3), 1.43 (3H, *s*, 18-CH_3), 1.43 (3H, *s*, 19-CH_3), 1.44 (2H, *m*, 3-CH_2), 1.50 (3H, *s*, 20-CH_3), 1.75 (2H, *m*, 2-CH_2), 1.78 (2H, *m*, 1-CH_2), 1.90 (2H, *m*, 2-CH_2), 1.93 (2H, *m*, 3-CH_2), 2.28 (2H, *m*, 1-CH_2), 3.19 (1H, *hept*, *J*=7.0 Hz, 15-CH), 5.78 (1H, *s*, 11-OH), 6.86 (1H, *s*, 6-CH), 7.15 (1H, *s*, 12-OH), 8.01 (1H, *s*, 14-CH); ^{13}C-NMR ($CDCl_3$): δ 17.6 (2-CH_2), 22.3 (16-CH_3), 22.5 (17-CH_3), 26.9 (15-CH), 27.6 (18-CH_3), 28.2 (19-CH_3), 33.6 (1-CH_2), 35.9 (4-C), 37.9 (3-CH_2), 40.3 (10-C), 111.4 (14-CH), 120.9 (8-C), 125.6 (6-CH), 133.8 (13-C), 141.0 (11-C), 143.8 (9-C), 154.9 (12-C), 157.7 (5-C), 179.7 (7-C).

*14-Deoxycoleon U (**5**, Figs. 1 and 2)* 182.8 mg. Pale yellow crystal. IR γ_{max} (KBr) cm^{-1}: 3523.3, 3380.6, 3232.1, 2962.1, 2935.1, 2875.3, 1768.4, 1631.5, 1583.3, 1554.3, 1488.8, 1467.6, 1411.6, 1342.2, 1270.9, 1187.9, 1135.9, 1060.7, 997.0, 908.3, 885.2, 784.9, 572.8, 464.8; mp: 210–212°C; EI-MS *m/z*: 330 (M^+, $C_{20}H_{26}O_4$, 35%), 315 (6), 287 (12), 274 (9), 262 (16), 261 (94), 260 (100), 248 (19), 247 (12), 245 (28), 233 (15), 232 (12), 231 (15), 219 (14), 217 (14), 191 (8), 128 (8), 115 (9), 82 (15), 77 (8), 69 (8), 55 (17); ^{1}H-NMR (pyridine-d_5): δ 1.30 (3H, *d*, *J*=7.0 Hz, 16-CH_3), 1.30 (3H, *d*, *J*=7.0 Hz, 17-CH_3), 1.64 (3H, *s*, 18-CH_3), 1.68 (3H, *s*, 19-CH_3), 1.95 (3H, *s*, 20-CH_3), 3.64 (1H, *hept*, *J*=7.0 Hz, 15-CH), 7.10 (1H, *s*, -OH), 8.24 (1H, *s*, 14-CH), 8.39 (1H, *s*, -OH); ^{13}C-NMR (pyridine-d_5): δ 18.3 (2-CH_2), 22.8 (16-CH_3), 23.1 (17-CH_3), 27.5 (19-CH_3), 27.6 (15-CH), 28.3 (18-CH_3), 28.4 (20-CH_3), 30.6 (1-CH_2), 36.7 (4-C), 36.9 (3-CH_2), 41.4 (10-C), 116.4 (14-CH), 121.6 (8-C), 135.6 (13-C), 140.3 (11-C), 142.4 (9-C), 144.2 (5-C), 144.3 (6-C), 150.2 (12-C), 180.7 (7-C).

*5,6-Dehydrosugiol (**6**, Figs. 1 and 2)* 21.3 mg. Pale yellow needle. IR γ_{max} (KBr) cm^{-1}: 2964.1, 2935.1, 2867.6, 1637.3, 1612.2, 1560.1, 1504.2, 1459.9, 1388.5, 1324.9, 1263.2, 1184.1, 891.0, 879.4, 869.7, 651.8; mp: 256°C; EI-MS *m/z*: 298 (M^+, $C_{20}H_{26}O_2$, 61%), 283 (25), 255 (32), 242 (14), 241 (16), 230 (35), 229 (84), 228 (42), 214 (18), 213 (100), 199 (34), 187 (30), 185 (10), 171 (11), 170 (12), 165 (15), 157 (13), 152 (11), 128 (11), 115 (12), 83 (10), 55 (23); ^{1}H-NMR (acetone-d_6): δ 1.18 (3H, *d*, *J*=7.0 Hz, 16-CH_3), 1.20 (3H, *d*, *J*=7.0 Hz, 17-CH_3), 1.21 (3H, *s*, 18-CH_3), 1.32 (3H, *s*, 19-CH_3), 1.47 (3H, *s*, 20-CH_3), 2.40 (2H, *m*, 1-CH_2), 3.23 (1H, *t*, *J*=1.7 Hz, 15-CH), 6.30 (1H, *s*, 6-CH), 6.95 (1H, *s*, 11-CH), 7.84 (1H, *s*, 14-CH); ^{13}C-NMR (acetone-d_6): δ 19.2 (2-CH_2), 22.6 (16-CH_3), 22.7 (17-CH_3), 27.5 (15-CH), 29.7 (20-CH_3), 32.8 (18-CH_3), 32.8 (19-CH_3), 38.0 (4-C), 38.5 (1-CH_2), 41.0 (3-CH_2), 41.9 (10-C), 111.4 (11-CH), 123.2 (8-C), 124.6 (6-CH), 124.9 (14-CH), 135.0 (13-C), 155.3 (9-C), 160.5 (12-C), 174.5 (5-C), 185.7 (7-C).

*Sandaracopimaric acid (**7**, Fig. 1)* 11.6 mg. Dark brown needle. IR γ_{max} (KBr) cm^{-1}: 2929.3, 2869.6, 1695.1, 1637.3, 1560.1, 1540.9, 1508.1, 1457.9, 1382.7, 1363.4, 1276.7, 997.0, 908.3; mp: 157–163°C; EI-MS *m/z*: 302 (M^+, $C_{20}H_{30}O_2$, 17%), 287 (30), 257 (8), 241 (8), 167 (15), 159 (9), 148 (12), 139 (22), 135 (21), 134 (18), 133 (28), 123 (25), 121 (100), 119 (28), 107 (34), 105 (33), 93 (42), 91 (51), 81 (30), 79 (43), 77 (24), 67 (27), 55 (45); ^{1}H-NMR ($CDCl_3$): δ 0.84 (3H, *s*, 20-CH_3), 1.04 (3H, *s*, 17-CH_3), 1.14 (2H, *m*, 1-CH_2), 1.21 (3H, *s*, 19-CH_3), 1.26 (2H, *m*, 6-CH_2), 1.36 (2H, *m*, 12-CH_2), 1.54 (2H, *m*, 11-CH_2), 1.60 (2H, *m*, 2-CH_2), 1.62 (2H, *m*, 3-CH_2), 1.66 (2H, *m*, 1-CH_2), 1.77 (1H, *m*, 9-CH), 1.93 (1H, *dd*, *J*=2.5 Hz, 12.4 Hz, 5-CH), 2.21 (2H, *d*, *J*=7.0 Hz, 7-CH_2), 4.89 (2H, *dd*, *J*=1.5 Hz, 10.4 Hz, 16-CH_2), 4.91 (2H, *dd*, *J*=1.5 Hz, 17.6 Hz, 16-CH_2), 5.22 (1H, *s*, 14-CH), 5.77 (1H, *dd*, *J*=10.5 Hz, 17.4 Hz, 15-CH); ^{13}C-NMR ($CDCl_3$): δ 15.5 (20-CH_3), 17.0 (19-CH_3), 18.4 (2-CH_2), 18.8 (11-CH_2), 24.9 (6-CH_2), 26.3 (17-CH_3), 34.7 (12-CH_2), 35.7 (7-CH_2), 37.3 (3-CH_2), 37.7 (10-C), 38.0 (13-C), 38.6 (1-CH_2), 47.6 (4-C), 49.1 (5-CH), 50.8 (9-CH), 110.4 (16-CH_2), 129.4 (14-CH), 136.9 (8-C), 149.2 (15-CH), 185.2 (18-COOH).

*Xanthoperol (**8**, Figs. 1 and 2)* 37.6 mg. Yellow needle. IR γ_{max} (KBr) cm^{-1}: 3371.0, 2961.2, 2938.0, 2903.3, 2871.5, 2361.4, 1715.4, 1655.6, 1592.0, 1566.9, 1466.6, 1328.7, 1290.1, 1263.2; mp: 246–249°C; EI-MS *m/z*: 314 (M^+, $C_{20}H_{26}O_3$, 28%), 286 (7), 272 (9), 271 (43), 229 (11), 217 (25), 205 (22), 204 (100), 203 (33), 187 (12), 173 (7), 161 (33), 128 (8), 115 (14), 91 (10), 77 (8), 69 (11), 55 (22); ^{1}H-NMR ($CDCl_3$): δ 0.46 (3H, *s*, 19-CH_3), 0.97 (3H, *s*, 18-CH_3), 1.22 (3H, *s*, 20-CH_3), 1.28 (2H, *s*, 1-CH_2), 1.28 (3H, *d*, *J*=7.0 Hz, 17-CH_3), 1.30 (3H, *d*, *J*=7.0 Hz, 16-CH_3), 1.31 (2H, *m*, 3-CH_2), 1.45 (2H, *m*, 3-CH_2), 1.57 (2H, *m*, 2-CH_2), 2.47 (2H, *d*, *J*=14.8 Hz, 1-CH_2), 2.64 (1H, *s*, 5-CH), 3.22 (1H, *hept*, *J*=7.0 Hz, 15-CH), 6.86 (1H, *s*, 11-CH), 8.04 (1H, *s*, 14-CH); ^{13}C-NMR ($CDCl_3$): δ 18.9 (2-CH_2), 22.3 (16-CH_3), 22.3 (17-CH_3), 24.1 (19-CH_3), 27.0 (15-CH), 31.4

Table 1 Termicidal activities of extracts from *Taxodium distichum* cones against *Reticulitermes speratus*

Fraction	Yields (%)	Mortality of termites[a]	
		5days	10days
Strong acidic	1.59	6.67±6.67	13.3±8.82
Medium acidic	1.39	10.0±5.77	33.3±20.3
Weak acidic	12.61	10.0±5.77	33.3±12.0
Neutral	84.41	n.m.[b]	6.67±3.33
Blank	-	3.33±3.33	3.33±3.33

[a] Concentration of compounds on paper disc (100×compound weight/paper disc weight) were 1.0%. *N*=3 replicates. 100%=30 termites. Means ± SE are given. Mortality rates were recorded every 24 hr during the test periods

[b] No mortality observed

(18-CH_3), 35.4 (4-C), 36.3 (1-CH_2), 38.5 (20-CH_3), 39.4 (10-C), 42.0 (3-CH_2), 68.9 (5-CH), 111.2 (11-CH), 127.2 (8-C), 129.8 (14-CH), 134.7 (13-C), 150.6 (9-C), 160.5 (12-C), 179.9 (7-C), 200.1 (6-C).

Results and Discussion

Bioactivities of the Compounds from T. distichum Cones The *n*-hexane soluble fraction (88.6 g) of *T. distichum* cones was separated by partition extraction and yielded strong (1.59%), medium (1.39%), and weak (12.61%) acidic fractions and a neutral fraction (84.41%). Termicidal activities of these four fractions were tested, and the results are shown in Table 1. The medium (33.3±20.3%) and the weak (33.3±12.0%) acidic fractions exhibited potent termicidal activity during a 10-d bioassay period. Based on the amount of extraction and the results of the bioassays, the weak acidic fraction seemed to contain the bioactive compounds. Therefore, it was further examined by silica gel column chromatography.

On a preparative scale, compounds **1**–**8** (Fig. 1) were isolated from the weak acidic fraction. The structures of these compounds, 6,7-dehydroroyleanone (**1**) (Hensch et al. 1975; Tezuka et al. 1998), taxodal (**2**) (Kusumoto et al. 2008), taxodione (**3**) (Kupchan et al. 1969; Tezuka et al. 1998), salvinolone (**4**) (Lin et al. 1989; Yamamoto et al. 2003), 14-deoxycoleon U (**5**) (Hueso-Rodriguez et al. 1983; Yamamoto et al. 2003), 5,6-dehydrosugiol (**6**) (Kupchan et al. 1969; Lin et al. 1989), sandaracopimaric acid (**7**) (Wenkert and Buckwalter 1972), and xanthoperol (**8**) (Kondo et al. 1963; Li et al. 2003), were determined by EI mass spectra, IR data, melting points, and NMR spectral data, and these were compared with published data. This is the first report of **2**, **7**, and **8** from *T. distichum* cones. Compounds **1**–**8**, except **2** and **7**, were diterpenes with an abietane-type structure.

Table 2 Antitermitic activities of compounds against *Reticulitermes speratus*

Compound	Mortality of termites[a]		Mass of paper fed by termites[d]	
	5days (%)	10days (%)	Mass (μg)[d]	Relative rate (%)[e]
6,7-Dehydroroyleanone (**1**)	46.7±3.33	70.0±10.0	8.97±1.91	20.3
Taxodal (**2**)	n.m.[b]	n.m.[b]	16.7±2.21	37.7
Taxodione (**3**)	n.m.[b]	20.0±11.6	2.97±0.72	6.7
Salvinolone (**4**)	3.33±3.33	16.7±6.67	9.33±0.86	21.1
14-Deoxycoleon U (**5**)	n.m.[b]	n.m.	0.83±0.55	1.9
5,6-Dehydrosugiol (**6**)	6.67±6.67	6.67±6.67	27.9±2.64	63.1
Sandaracopimaric acid (**7**)	n.t.[c]	n.t.[c]	n.t.[c]	n.t.[c]
Xanthoperol (**8**)	6.67±3.33	10.0±5.77	0.0	0.0
Ferruginol (**9**)	10.0±5.77	20.0±11.6	0.44±0.19	1.0
6,7-Dehydroferruginol (**10**)	n.m.[b]	3.33±3.33	7.35±2.56	16.6
Sugiol (**11**)	3.33±3.33	3.33±3.33	41.9±2.33	94.7
No feed	3.33±3.33	13.3±3.33	-	-
Blank	1.67±1.67	1.67±1.67	44.3±1.54	100

[a] The concentration of the sample in the dried paper discs was prepared to 1.0% (sample weight/paper disc weight×100). *N*=3 replicates. 100%=30 termites. Means ± SE are given. Mortality rates were recorded every 24 h during the test periods

[b] No mortality observed

[c] None tested

[d] The mass of paper discs fed by one termite per 24 hr (calculated by mass loss of paper disc in 10 d divided by total number of feeding termites in 10 d)

[e] Relative rates were calculated from blank reading (100×mass of each sample/mass of blank)

The termicidal and antifeedant activities of compounds **1**–**8** against *R. speratus* were tested, (except for **7**, which was isolated in too low amounts), together with ferruginol (**9**), 6,7-dehydroferruginol (**10**), and sugiol (**11**). The results are shown in Table 2. Compounds **9**–**11** are well-known abietane-type diterpenes that exist in Taxodiaceae (Otto et al. 2002). Compound **9** has been shown previously to have antitermitic and antifungal activities (Kofujita et al. 2001; Kano et al. 2004).

When testing termicidal activity, **1** caused strong mortality (46.7±3.33% in 5 d, 70.0±10.0% in 10 d). Compound **3** also caused notable mortality after 10 d (20.0±11.6%), with the same value as **9**. The mortality rates caused by **8** after 10 d (10.0±5.77%) and the rates observed in the "no feed" assay (13.3±3.33%) were similar.

When testing antifeedant activity, **8** showed effective activitiy against *R. speratus*. The termites fed on paper treated with **8**. Compounds **3** and **5** also showed potent relative activities in the antifeedant test.

From these results, we conclude that *T. distichum* cones contain strong bioactive compounds (**1**, **3**, **5**, and **8**). In particular, **1** is a specific bioactive compound in the *n*-hexane extract of *T. distichum* cones, and has a quinone structure at the C ring (Fig. 2). The abietane-type diterpenes, which have quinone structures at the C rings, such as cryptoquinone and 7-hydroxy-11,14-dioxo-8,12-abietadiene, previously have been reported to show anti-fungal and cytotoxic activities (Kofujita et al. 2002, 2006). Herein, we report that **1**, the abietane-type quinone, exhibited strong activities against subterranean termite.

Bioactivities of the Oxidized Abietane-type Compounds It was assumed that the bioactive compounds **1**–**8** were oxides of **9** (Yamamoto et al. 2003). In order to compare the bioactive value of **9** with its oxides, the oxidation routes of **9** and the relative values on the basis of **9**, as a standard compound, are indicated in Fig. 2.

First, we discuss the termicidal activity (Fig. 2a). The activity decreased with dehydrogenation of C-6 and C-7 at **10**, but the activity increased with the synthesis of a quinone structure in the oxidation progression to **1** or **3**. Activity also decreased at **11**, but increased again through oxidation to **4**, **6**, and **8**. Moreover, oxidation of **4** to **5** resulted in loss of all activity.

Second, a similar change was observed with antifeedant activity (Fig. 2b). The activity of **3** was stronger than that of **1**. Furthermore, the activity completely disappeared at **11**, but high activities were obtained through oxidation to **5** or **8**.

These findings suggest that the various forms of the abietane-type structure due to the degree of oxidation reflect their various bioactivities. As several compounds that are active against *R. speratus* were discovered, they may be related to the defense of *T. distichum*. Hence, we consider that this may be one of the important factors that explains why *T. distichum* have prospered on the earth.

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The Biogeographical Distribution of Duncecap Larkspur (*Delphinium occidentale*) Chemotypes and Their Potential Toxicity

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Abstract Larkspurs (*Delphinium* spp.) are poisonous plants found on rangelands in western North America. Larkspur's toxicity has been attributed to the norditerpenoid alkaloids, which are divided into two main structural groups: the highly toxic (*N*-methylsuccinimido) anthranoyllycoctonine type (MSAL type) and the less toxic 7,8-methylenedioxylycoctonine type (MDL type). Plants high in the MSAL-type alkaloids are thought to be the most toxic to cattle, and the concentrations of these alkaloids have been used as a predictor of plant toxicity. Duncecap larkspur, *Delphinium occidentale*, occurs throughout much of the Intermountain West and Northwestern United States. Specimens from field collections and herbaria deposits were evaluated taxonomically and chemically. Two distinct alkaloid profiles were identified: one that contains the MSAL-type alkaloids and one that contains little, if any, MSAL-type alkaloids. Thus, plants with these two alkaloid profiles should differ in their toxic potential. Each profile was unique in its geographical distribution. These findings have important implications in grazing management decisions on *D. occidentale*-infested rangelands, and they demonstrate that botanical classification alone is not a good indicator to determine the toxic risk of *D. occidentale*.

Keywords Larkspur · MSAL-type alkaloids · Norditerpene · *Delphinium occidentale* · Methyllycaconitine

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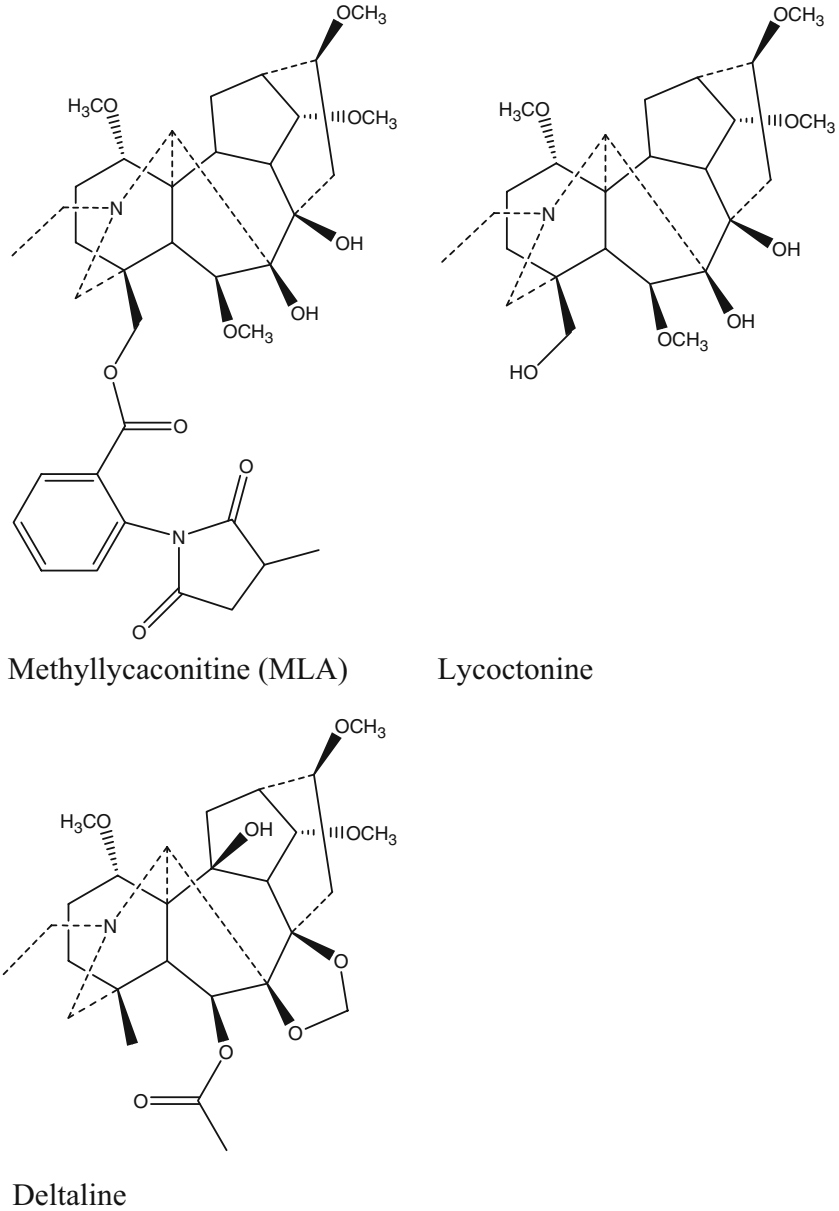
Introduction

Larkspurs (*Delphinium* spp.) are poisonous plants on rangelands in the western USA. They are responsible for significant losses to the cattle industry and are the subject of extensive research (Marsh et al. 1916, 1934; Pfister et al. 1999, 2002). Total cost to the livestock industry from cattle deaths attributed to larkspur poisoning is estimated to be millions of dollars annually (Nielsen et al. 1994). Larkspurs are divided into three groups based principally upon their height: low larkspurs, plains larkspurs, and tall larkspurs. The tall larkspurs are responsible for a greater number of cattle losses than either the plains or low larkspur.

Larkspur-induced poisoning in cattle is attributed to the diterpenoid alkaloids that can represent up to 3% of the plant dry weight. There are two main structural groups of norditerpene alkaloids, the *N*-(methylsuccinimido) anthranoyllycoctonine type (MSAL-type) and the 7,8-methylenedioxylycoconine type (MDL-type) norditerpenoid alkaloids (Fig. 1; Olsen et al. 1990). The MSAL-type are approximately 20 times more toxic than the MDL-type based upon the LD_{50} of the individual compounds in a mouse model (Manners et al. 1993, 1995; Panter et al. 2002). Acute larkspur poisoning has been attributed to the MSAL-type alkaloids (Aiyar et al. 1979; Pfister et al. 1999), and plants high in the MSAL type are thought to be the most toxic to cattle. The concentrations of these alkaloids have been used as a predictor of plant toxicity (Pfister et al. 2002; Ralphs et al. 2002). The most abundant member of the MSAL-type alkaloids in the tall larkspurs is methyllycaconitine (MLA; Gardner et al. 2002).

Two synopses of the genus *Delphinium* found in North America have been published, the first by Ewan (1945) and the second by Warnock (1995, 1997). Three tall larkspur species (*Delphinium barbeyi*, *Delphinium occidentale*, and

Fig. 1 Structure of select norditerpene alkaloids in *Delphinium occidentale*



Delphinium glaucum) of particular interest received different taxonomic treatment by both authors. Recent research from standard systematics (Welsh and Ralphs 2002), chemotaxonomy (Gardner et al. 2002), and molecular markers (Li et al. 2002) support the treatment given to these three species by Ewan (1945). All of these species can contain the MSAL-type alkaloids with MLA being the major member.

An observation of particular interest made by Gardner et al. 2002 was the identification of two alkaloid profiles in *D. occidentale*. One alkaloid profile lacked or displayed small amounts of the MSAL-type alkaloids, whereas the other profile displayed large amounts of the MSAL-type alkaloids. The objective of this study was to determine the extent of these two profiles throughout the geographical distribution of *D. occidentale*. We report here that *D. occidentale* has two definable chemotypes, with distinct geographical boundaries, that should differ in potential toxicity. These results have implications for grazing management decisions in *D. occidentale*-infested rangelands, and they demonstrate that taxonomic classification alone is not a good indicator to determine the toxic risk of *D. occidentale*.

Methods and Materials

Plant Materials Analytical samples were prepared from plant material collected from herbarium specimens and resident populations of *D. occidentale*. Herbarium specimens were provided by the Intermountain Herbarium at

Utah State University, the Stanley L. Welsh Vascular Plant Herbarium at Brigham Young University, the University of Colorado Museum Herbarium, the Rocky Mountain Herbarium at the University of Wyoming, the University of Washington Herbarium, and the Herbarium at Oregon State University. Specimens of questionable identification were verified to be authentic *D. occidentale* specimens by staff at the Intermountain Herbarium at Utah State University or the Stanley L. Welsh Vascular Plant Herbarium at Brigham Young University. Leaf and flower material were sampled from herbarium specimens and subsequently ground by using a Retsch mixer mill MM301 (Haan, Germany).

Field samples of *D. occidentale* populations (1,464 plants representing 118 accessions) were collected in the summer of 2007 and 2008. Accessions were collected throughout the geographical distribution of *D. occidentale* including the states of Utah, Idaho, Montana, Wyoming, Colorado, Nevada, and Oregon. Samples immediately were placed on dry ice after collection and stored at −80°C upon return to the laboratory. Samples were frozen for possible use in subsequent research. Samples were freeze-dried and ground to pass through a 2-mm screen using a Wiley mill.

Sample Extraction and Alkaloid Analysis Individual plant samples were extracted and analyzed by electrospray mass spectrometry by using procedures previously described (Gardner et al. 1999). In summary, 25-mg plant material from herbarium samples was extracted in 6 ml of methanol for 16 h. Reserpine (125 µg) was added as an internal reference. The sample was mixed then centrifuged. A 200-µl sample was diluted into 800 µl of 1:1 methanol/1% acetic acid. For plant samples collected in the field in the summers of 2007 and 2008, 100-mg plant material was extracted in 6 ml of methanol for 16 h. Reserpine (500 µg) was added as an internal reference. The sample was mixed then centrifuged. A 50-µl sample was diluted into 950 µl of 1:1 methanol/1% acetic acid.

Mass spectra were recorded for each sample over a range of 150–800 *m*/*z* and averaged across all scans taken at 40% of peak height (total ion current). Data were calculated by recording the abundance of all ions above a relative area of 0.1%. The amount of a compound (as represented by a single mass unit) detected was calculated based on the relative abundance of the internal standard reserpine (MH^+=609). The resulting mass spectral data were reduced and tabulated to a final set of quantitative values for 57 different protonated molecules by using a method similar to that reported by Gardner et al. (2002) (Table 1).

Data Analysis Each sample was assigned into group A (samples with MLA concentrations greater than 100 µg/mg) or group B (samples with MLA concentrations less than 100 µg/mg). This cutoff was chosen because it clearly separated the two alkaloid profiles observed previously by Gardner et al. (2002). MANOVA and discriminant analysis of the two assigned groups were performed as a pairwise comparison by using BioNumerics 4.6 (Applied Maths, Inc.). Two parameters were reported: (1) *L* (Wilk's Lambda likelihood ratio test) is the likelihood of the obtained discrimination with the assumption that the groups are drawn from the same population. A low *L* value infers that the groups are likely to be drawn from different populations. (2) *P* is the probability that a random grouping of the groups would yield the same degree of discrimination. *t* tests were performed by using Microsoft Excel. All multivariate and univariate statistical comparisons were made from plants of the same developmental stage.

Results

Field collections (1,464 specimens representing 118 accessions) of *D. occidentale* were made in the summer of 2007 and 2008. Samples were representative of the reported geographical distribution of *D. occidentale.* In addition, 599 herbarium specimens of *D. occidentale* from the cooperating herbaria were sampled. These specimens were collected from the late 1800s to the current year, and they also were representative of the reported geographical distribution of *D. occidentale*. All samples were analyzed by electrospray mass spectrometry. The resulting mass spectral data were reduced and tabulated to a final set of quantitative values for 57 different masses (Table 1).

Initially, each sample was scored for the presence of MLA (>100 µg/mg) or reduced amounts of MLA (<100 µg/mg). A total of 698 samples were identified that contained greater than 100 µg/mg MLA. A total of 1,365 samples were identified that contained less than 100 µg/mg MLA. To confirm that these two groups were unique, multivariate statistical methods (MANOVA and discriminant analysis) were used to test for grouping. The pairwise MANOVA revealed that the two groups were different (*P*= 0.001%, *L*=0.15). Discriminant analysis also was performed by comparing the two groups as a pairwise comparison. Discriminant analysis showed clear separation of the two groups based upon multiple alkaloids. The five most important discriminants were the following masses: *m*/*z* 683 (MLA), 715, 739, 753, and 699. These two multivariate methods demonstrated that the two groups were different. As a result, samples with greater than 100 µg/mg MLA will hereafter be termed chemotype A and those samples that have less than 100 µg/mg MLA will hereafter be termed chemotype B. Representative mass

Table 1 Comparison of total alkaloid extract from *Delphinium occidentale* chemotypes A and B

m/z[a]	Possible alkaloid(s)[b]	Chemotype A[c]	Chemotype B[c]
365	Unknown	17	16
386*	Delnuttaline, geyeridine	9	14
397	Unknown	16	16
404*	Unknown	65	46
414	11,13-Diacetyhetisine	15	20
428*	Geyerine	1	54
432*	Unknown	15	26
434*	8-Acetylkarasamine	9	15
436*	14-Acetylleroyine, delelatine	22	28
446	Unknown	54	88
450*	Delpheline, occidentaline	15	28
452*	Dictyocapinine, chasmanine	13	38
454*	Delcosine, delectinine	23	77
464*	6-Dehydrodeltamine	11	21
466*	Deltamine, 14-dehydrobrowniine	56	89
468	Delsoline, lycoctonine	190	218
476*	Unknown	41	58
480*	Barbeline, 14-acetyldelcosine	11	23
482*	Delphatine	50	165
492	Barbinidine	19	23
494	Dictyocarpine	180	175
496*	14-Acetyldelectinine, 6-epi-pubescenine	17	32
498*	Unknown	26	59
500	Unknown	7	4
504*	Unknown	5	11
508*	Deltaline	739	1120
510*	14-Acetylbrowniine	56	114
524*	Unknown	14	30
526	Unknown	1	4
527	Unknown	9	9
536	14-Acetlydictyocarpine	33	23
538	Unknown	36	25
546	Unknown	9	13
550	Unknown	30	31
552	Glaucedine	245	183
564	Glaucerine	52	72
568	Unknown	4	5
578	Barbisine, glaucenine	163	307
587*	Anthranolyllycoctonine	10	0
598*	Glaucephine	16	26
633*	Unknown	15	5
641	Unknown	24	23
649	Unknown	11	10
665*	Unknown	14	3
667*	Barbinine	11	1
669*	14-Deacetylnudicauline, 16-deacetylgeyerline	34	3
675*	Unknown	9	2
683*	Methyllycaconitine	656	4
689	Unknown	6	4

Table 1 (continued)

m/z[a]	Possible alkaloid(s)[b]	Chemotype A[c]	Chemotype B[c]
691*	Unknown	21	6
697*	Unknown	3	0
699*	Unknown	14	1
711*	Geyerline, nudicauline, acetylgrandiflourine	38	1
715*	Unknown	13	1
739*	Unknown	21	1
753*	Unknown	45	1
773*	Unknown	9	1

[a] Masses followed by an asterisk are different between the two chemotypes ($P<0.05/57=0.0009$). Analyses were performed using a *t* test with pairwise comparison of means

[b] Alkaloid identification taken from Pelletier et al. (1981, 1989), Joshi et al. (1988, 1989), Kulanthaivel et al. (1988, 1990), and Manners et al. (1996, 1998)

[c] Relative alkaloid concentrations per 100 mg of plant material. Concentrations were normalized to an internal reserpine standard

spectra of the two chemotypes are shown in Fig. 2. Chemotype A contains the MDL- and MSAL-type alkaloids including MLA, while chemotype B contains principally the MDL-type alkaloids and very little, if any, MLA.

A *t* test was used to identify masses whose concentrations are different between the two chemotypes. The mean concentration of each mass for each chemotype and the possible alkaloid each mass may represent are listed in Table 1. A conservative approach was taken for identifying masses whose concentrations are different by using the Bonferroni *P* value at $\alpha=0.05/57=0.0009$. By using these criteria, 36 masses were identified whose concentrations were different between the two chemotypes. The concentrations of 16 masses were greater in chemotype A and the concentrations of 20 were greater in chemotype B. The alkaloids that accumulated to greater amounts in chemotype A were other MSAL-type alkaloids in addition to MLA, while those alkaloids that accumulated to greater amounts in chemotype B were the MDL-type alkaloids.

The geographical distribution of each chemotype was investigated by state and counties within each state. Table 2 shows the number of samples analyzed from each state, county, and the number of samples assigned to each chemotype. A distribution map of the two chemotypes is shown in Fig. 3. Most counties had only samples of one chemotype. However, a number of counties such as Caribou (ID), Lincoln (WY), Sublette (WY), and Fremont (WY) have samples of each chemotype, indicating that they serve as a transition zone from south to north. Although these counties have plants with both chemotypes, individual populations from field collections in each county were either chemotype A or B in most cases. Likewise, counties that are in the transition zone from east to west, such as Juab and Box Elder counties of Utah, also have samples of both chemotypes but are spatially separated: chemotype A to the west and chemotype B to the east.

Discussion

Previous research has demonstrated that the MSAL-type alkaloids are more toxic than the MDL-type in a mouse model (Manners et al. 1993, 1995; Panter et al. 2002; Welch et al. 2008). The MSAL-type alkaloids are thought to be the most toxic to cattle, and their concentrations have been used as a predictor of plant toxicity (Ralphs et al. 2002; Pfister et al. 2002). Consequently, current management recommendations for grazing cattle on larkspur containing ranges are based primarily on the concentration of MSAL-type alkaloids (Pfister et al. 2002; Ralphs et al. 2002).

In considering these facts, a finding of particular interest made by Gardner et al. (2002) was the identification of two alkaloid profiles in *D. occidentale*. One profile lacked or displayed small amounts of the MSAL-type alkaloids, and another that displayed larger amounts of the same alkaloids. However, no information regarding the relative distribution of these two profiles was provided. Therefore, the objective of this study was to define the distribution of these two profiles throughout the geographical distribution of *D. occidentale* by using field collections and herbarium specimens.

Two chemotypes of *D. occidentale* were identified, thus supporting the preliminary finding of Gardner et al. (2002). Chemotypes A and B contain similar MDL-type alkaloids but at different concentrations (Table 1). However, no MDL-type alkaloid was chemotype specific, and differences in the MDL-type alkaloids between chemotypes were due to the large sample size. Localized populations of each chemotype may not contain statistically different amounts of the MDL-type alkaloids. The major MDL-type alkaloids in both chemotypes based upon concentration are those with masses (*m/z*) 454, 468, 482, 494, 508, 552, and 578 (Table 1). Deltaline ($MH^+=508$) is the major MDL-type alkaloid in most samples of both chemotypes; however, the

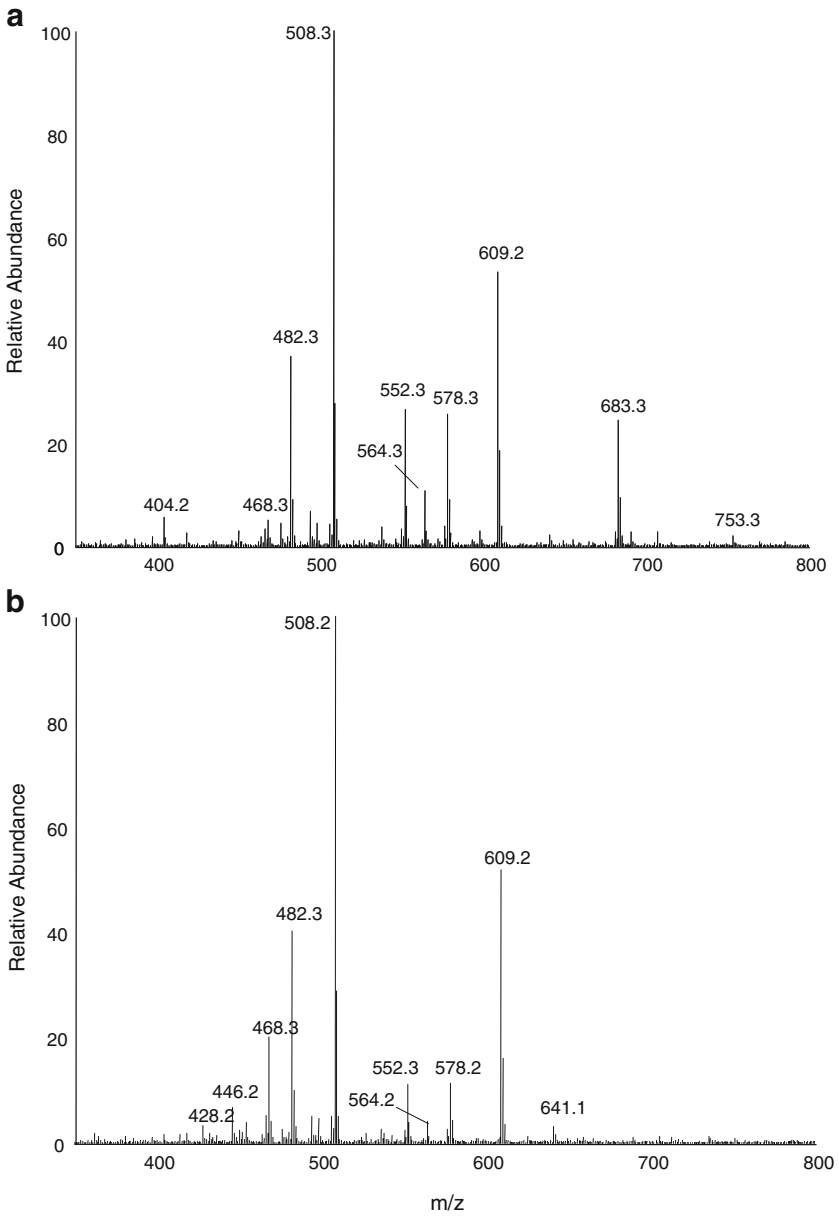


Fig. 2 Electrospray mass spectra from representative samples from each *Delphinium occidentale* chemotype

ratio of deltaline to other MDL alkaloids varies among locations. For example, in some locations masses (*m*/*z*) 454, 552, and 578 may be the major MDL-type alkaloid. Chemotypes A and B contain different concentrations of the MSAL-type alkaloids. Concentrations of the other MSAL-type alkaloids such as nudicauline, geyerline, 14-deacetynudicauline, and others are different between the two chemotypes but like the MDL-type alkaloids are not chemotype specific. This is due to the fact that some of these minor MSAL-type alkaloids are not found in all populations of chemotype A. Methyllycaconitine (MH^+= 683) concentration, which was used as the basis for assigning the two groups, was chemotype specific by definition. Three conclusions can be drawn from these data.

First, plants that represent chemotype B containing very low amounts or no detectable MSAL-type alkaloids would likely pose little risk to grazing cattle based upon current models and recommendations. Current management recommendations state that plants with greater than 3-mg MSAL-type alkaloids per gram plant material pose the greatest risk to cattle (Pfister et al. 2002). Alternatively, plants that represent chemotype A containing the MSAL-type alkaloids may pose considerable risk to cattle based upon current models and recommendations. However, poisoning is always dependent upon the dose and duration. Chemotype B plants contain the less toxic MDL-type alkaloids. Therefore, at the proper dose and duration, there is a possible risk of poisoning livestock.

Table 2 The biogeographical distribution of each *Delphinium occidentale* chemotype by state and county

State	County	Total	Chemotype A	Chemotype B
Colorado	Garfield	3		3
	Grand	8		8
	Jackson	19		19
	Pitkin	1		1
	Rioblanco	16		16
	Routt	26		26
Idaho	Bannock	15	12	3
	Bear Lake	89		89
	Blaine	7	7	
	Boise	5	5	
	Bonneville	31	31	
	Canyon	1	1	
	Caribou	67	7	60
	Cassia	19	19	
	Clark	9	8	1
	Clearwater	7	7	
	Custer	13	13	
	Elmore	1	1	
	Franklin	00	12	88
	Fremont	32	32	
	Idaho	4	4	
	Lemhi	15	15	
	Madison	2	2	
	Payette	2	2	
	Teton	9	9	
	Twin Falls	20	20	
	Valley	1	1	
Montana	Beaverhead	45	44	1
	Gallatin	55	54	1
	Madison	26	26	
	Park	35	34	1
	Stillwater	2	2	
	Sweet Grass	10	10	
Nevada	Elko	58	58	
	Eureka	6	6	
	White Pine	20	20	
Oregon	Baker	2	2	
	Union	9	9	
	Wallowa	15	15	
Utah	Box Elder	34	26	8
	Cache	303		303
	Carbon	2		2
	Daggett	14		14
	Davis	1		1
	Duchesne		10	10
	Emery	14		14
	Juab	44	8	36
	Millard	38		38
	Rich	15		15
	Salt Lake	31	1	30
	Sanpete	89		89
	Sevier	38		38
	Summit	58		58
	Tooele	16		16
	Uintah	22		22
	Utah	52	2	50
	Wasatch	67		67
	Weber	23		23
	Albany	30		30
Wyoming	Carbon	45		45
	Converse	1		1
	Fremont	10	6	4
	Hot Springs	1		1
	Lincoln	87	28	59
	Natrona	2		2
	Park	26	25	1
	Sublette	90	27	63
	Sweetwater	2		2
	Teton	92	87	5
	Uinta	1		1

Second, in general, each chemotype had a distinct distribution with defined boundaries (Table 2 and Fig. 3). In some cases, the chemotypes are separated by notable geographic features. For example, the east to west transition is separated by the desert that runs north to south on the west side of the state of Utah (Fig. 3). On the other hand, the north to south transition zone of the two chemotypes is not separated by any notable geographic features. In fact, two populations in Lincoln County, Wyoming that represent the two different chemotypes occurred on the same watershed less than 5 miles apart.

Third, the data suggest that the qualitative nature of the alkaloid profiles in *D. occidentale* remains constant at a given location. This conclusion is supported by field collections that have the same qualitative alkaloid profiles as the herbarium specimens from identical locations that were collected up to 100 years earlier. In addition, the data suggest that the alkaloid composition of herbarium specimens is not modified as a result of long-term storage at room temperature. Thus, herbarium specimens may serve as useful resources in determining risk of other larkspur species. However, quantitative amounts of these alkaloids may vary among years, and quantitative assessment of the alkaloids over time due to environment and other factors merits further investigation. For example, in *D. barbeyi*, a closely related tall larkspur species, both the MDL- and MSAL-type alkaloids decline as the plant matures and

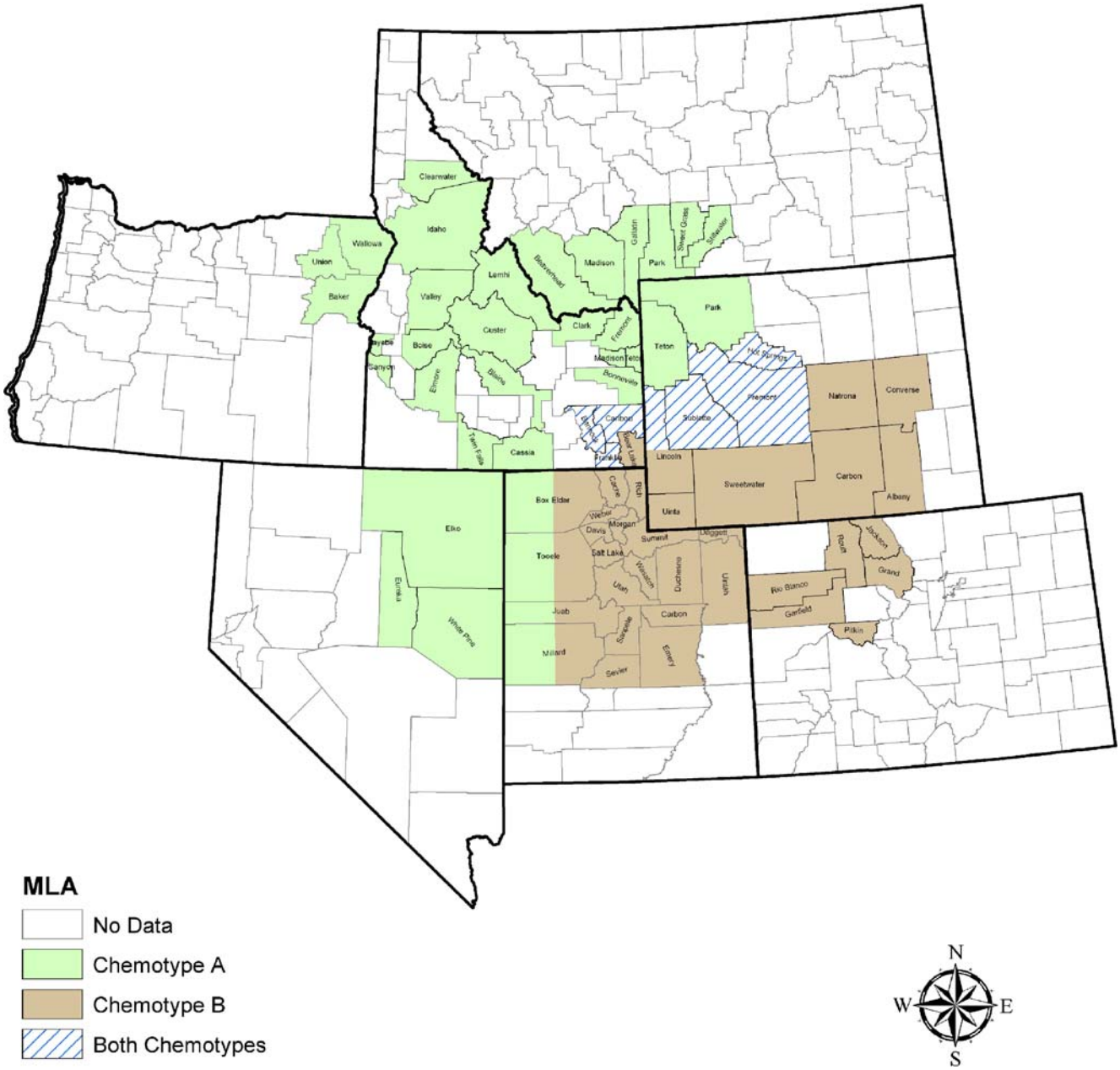


Fig. 3 Distribution map of chemotypes A and B throughout the geographical distribution of *Delphinium occidentale*

accumulate to different amounts in differing plant tissues (Ralphs and Gardner 2003). Additionally, environmental factors such as light, defoliation, and herbivory by insects have been shown to influence norditerpene alkaloid concentrations and pools (Ralphs et al. 1998a, b; Ralphs and Gardner 2001b). Such causes could be responsible for or play some part in the observed differences between the two chemotypes as well as their distribution.

We currently are not able to explain why there are two unique chemotypes of *D. occidentale* with such a defined geographical distribution, although some possibilities merit consideration. First, these chemotypes may represent unique *Delphinium* species or varieties, although no obvious morphological features used for taxonomic separation of the two groups have been noted. Second, the chemotypes may have originated due to environmental differences among locations. Previous research has shown that *D. occidentale* with chemotype A and *D. barbeyi* have distinct alkaloid profiles. However, when reciprocal gardens were established with these two species, the variation in profiles disappeared (Ralphs and Gardner 2001a). Third, these chemical profiles may be the result of natural phytochemical evolution of plants in the presence of herbivores. Frequency-dependent selection by herbivores may result in the evolution of differing chemical phenotypes (Schmeller et al. 1994). Furthermore, in certain

instances, plants with one chemotype have been shown to be more susceptible to herbivory than those of another chemotype (Berenbaum and Zangerl 1988; Bowers and Puttick 1988). It appears likely that plants with chemotype B may be more susceptible to herbivory due to their lack of methyllycaconitine, a potent insecticide (Jennings et al. 1986). Finally, chemotype B may be derived from hybridization between chemotype A of *D. occidentale* and *D. barbeyi* as suggested previously (Ralphs and Gardner 2001a; Gardner et al. 2002; Li et al. 2002). In fact, chemotype B may overlap with *D. barbeyi* in parts of Sevier, Sanpete, and Emery counties in Utah, all the counties in Colorado, and Albany and Carbon counties in Wyoming (Ralphs and Gardner 2001a; Gardner et al. 2002). Hybridization can result in either the gain of novel secondary compounds or the loss of secondary compounds (Orians 2000). If chemotype B were derived from hybridization, it is possible that a loss of function in the biosynthetic pathway leading to the MSAL-type alkaloids occurred, which could result in the accumulation of the MDL-type alkaloids. To address such possibilities, we are initiating further studies that include phylogenetic analysis and reciprocal gardens.

In summary, the MSAL-type alkaloids, such as MLA, are the primary factors responsible for the toxicity of larkspur plants. We report here that *D. occidentale* has two defined chemotypes: one (chemotype A) contains significantly more MSAL-type-alkaloids, and one lacks or contains very small amounts of the MSAL-type alkaloids (chemotype B). In general, the plants with these chemotypes grow in distinct geographical locations; however, there are counties that contain populations of both chemotypes. In addition, this study demonstrates that taxonomic identification of *D. occidentale* is not sufficient to determine risk. Lastly, based upon current toxicity models, the results from this study have implications for making management decisions for D. *occidentale*-infested rangelands. Additional research, however, is needed to determine the exact risk to livestock of each chemotype before these management recommendations can be further refined.

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RAPID COMMUNICATION

Drones of the Dwarf Honey Bee *Apis Florea* Are Attracted to (2E)-9-Oxodecenoic Acid and (2E)-10-Hydroxydecenoic Acid

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Abstract The queen mandibular gland component (2E)-9-oxodecenoic acid (9-ODA) has been suggested to function as the major sex pheromone component in all honey bee species. In contrast to this hypothesis, chemical analyses showed that in the Asian dwarf honey bee species, *Apis florea*, a different decenoic acid, (2E)-10-hydroxydecenoic acid (10-HDA), is the major component in the mandibular gland secretion. We show here that *A. florea* drones are attracted to 9-ODA as well as to 10-HDA. However, 10-HDA attracted higher numbers of drones at lower dosages than 9-ODA, and also was more attractive when directly compared to 9-ODA in a dual attraction experiment. We conclude that 10-HDA has to be viewed as the major sex pheromone in *A. florea*. The result that both pheromone components are capable of attracting drones when presented alone was unexpected with regard to existing sex pheromone attraction experiments in honey bees.

Keywords Sex pheromone · Mandibular gland · (2E)-9-oxodecenoic acid · (2E)-10-hydroxydecenoic acid · *Apis florea* · Honey bee

Introduction

Sex pheromone communication in honey bees is thought to be similar among the different species, and the general idea is that all species use (2E)-9-oxodecenoic acid (9-ODA), a queen mandibular gland component, as the major sex pheromone (Koeniger and Koeniger 2000). However, chemical analyses of queen mandibular gland secretions have shown that 9-ODA is not the major component in all species and all virgin queens (Plettner et al. 1997; Keeling et al. 2000). Virgin and mated *Apis florea* queens as well as virgin queens of the Western honey bee, *Apis mellifera*, synthesize higher amounts of (2E)-10-hydroxydecenoic acid (10-HDA) than 9-ODA (Plettner et al. 1997). In *A. mellifera*, 9-ODA attracts drones when presented alone, and 10-HDA increases the attractiveness of 9-ODA (Brockmann et al. 2006). Electroantennogram recordings and gene expression analysis showed a specialization of the drone olfactory system to detect 9-ODA (Brockmann et al. 1998.Wanner et al. 2007). Reports on sex pheromone communication in *A. florea* are contradictory. Sannasi et al. (1971) reported evidence that *A. florea* drones are attracted to 9-ODA, whereas other authors stated that attempts to attract *A. florea* drones with 9-ODA had failed (see Oldroyd and Wongsiri 2006). Because there is a close match between the signals produced by the sender and the behavioral responsiveness of the receiver in animal communication systems, we decided to test whether drones of *A. florea* are attracted to 9-ODA or 10-HDA.

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Methods and Materials

The drone attraction experiments were conducted at Gandhi Krishi Vignan Kendra (GKVK) campus of the University of Agricultural Sciences, Bangalore, South India during March 2006. Synthetic samples of (2E)-9-oxodecenoic acid (9-ODA) and (2E)-10-hydroxydecenoic acid (10-HDA) were purchased from Phero Tech Inc. (Delta BC, Canada). At first, we performed attraction experiments to generate

dose response curves for each pheromone component. Altogether, we tested eight dosages of 9-ODA (0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2 mg/ml) and five dosages of 10-HDA (0.1, 0.3, 0.5, 0.75, and 1.0 mg/ml). Dilution of 9-ODA and 10-HDA were prepared with absolute ethanol according to the manufacture's protocol. We used cotton wicks as pheromone dispensers, which were impregnated with 1 ml of the respective pheromone solution. Baits were lifted about 3 m into the air with a helium-filled balloon at known drone congregation areas (Nagaraja and Brockmann unpubl.). Each pheromone dosage was tested in 3–5 attraction experiments, and presentation of pheromone components and dosages were randomized. In all experiments, drone attraction was measured as the number of drones counted in the vicinity of the pheromone bait during 10 min by using binoculars. In a second series of experiments (N=6), we compared directly the attractiveness of 9-ODA vs. 10-HDA (and as a control 10-HDA vs.10-HDA) using the concentrations that attracted the highest number of drones in the dose response experiments (1.5 mg/ml for 9-ODA; 0.5 mg/ml for 10-HDA). The two baits were attached to separate helium filled balloons flying in the air about 2.0 to 2.5 m apart. Finally, to confirm that our visual identification of *A. florea* drones was correct, we performed control experiments (N=6) in which we compared the number of drones visually observed with the number of drones caught when presenting 9-ODA (1.5 mg/ml) or 10-HDA (0.5 mg/ml). For this purpose, we attached pheromone impregnated cotton wicks to glue plates (Trubble gum®, Pest control India Ltd., Bangalore, India). Although we caught a significantly lower number of drones with the glue plates than we had visually observed, this control experiment confirmed that the visual identification of *A. florea* drones was correct (see Fig. 1d).

All experiments were done over a period of several days. Each experimental day we conducted 5–10 attraction experiments during the daily drone flight time from 11:30 to 14:00 h. Results of the dose response curves were analyzed statistically by using a one-way ANOVA and a Tukey *post hoc* test for pair wise comparisons, and results of the dual attraction experiments were analyzed by using a paired t-test.

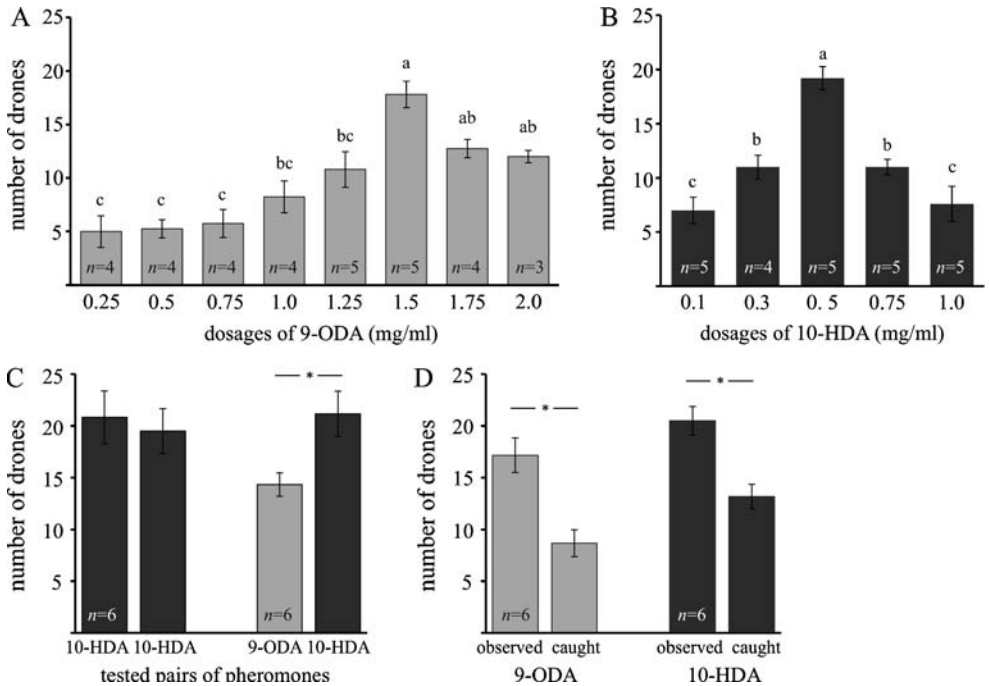

Fig. 1 **a** Dose-response curve for 9-ODA. **b** Dose-response curve for 10-HDA. 9-ODA attracted a maximum number of drones (17.8±1.2 SE) at dosages of 1.5–2.0 mg/ml (one-way ANOVA, df=7, F=12.481, P<0.001). In contrast, 10-HDA attracted a similar maximum number of drones (19±1.1 SE) at a dosage of 0.5 mg/ml (one-way ANOVA, df=4, F=17.295, P<0.001; Tukey test P<0.05). The highest dosage of 10-HDA led to a decrease in the number of drones attracted (Tukey test P<0.05). **c** Dual attraction experiment comparing attractiveness of 9-ODA (1.5 mg/ml) and 10-HDA (0.5 mg/ml). A lower dosage of 10-HDA attracted a significantly higher number of drones than a higher dosage of 9-ODA (paired t-test: t=−2.64, df=5, P two-tailed <0.05). **d** Single component attraction experiments comparing number of drones visually observed and caught. Number of drones, which were caught with the glue plate, were significantly lower that number of drones, which were visually observed close to the glue plates for both pheromone components (9-ODA: paired t-test: t=4.0194, df=10, P two-tailed <0.01; 10-HDA: paired t-test: t=4.0100, df=10, P two-tailed <0.01). Columns indicate average number of drones (±SE), grey columns are responses to 9-ODA, black columns are responses to 10-HDA; N indicates number of pheromone attraction experiments

Results and Discussion

Both mandibular gland components, 9-ODA and 10-HDA, attracted drones when presented alone. However, 10-HDA attracted a maximum number of drones at lower dosages than 9-ODA in the dose response curve experiments (Fig. 1a/b), and 10-HDA also attracted higher numbers of drones than 9-ODA when we directly compared the most attractive dosages of both components (Fig. 1c). On the basis of these results, we conclude that 10-HDA has to be viewed as the major sex pheromone component in *A. florea*. This finding correlates nicely with the fact that *A. florea* queens synthesize more 10-HDA than 9-ODA (Plettner et al. 1997; Keeling et al. 2000).

The result that 10-HDA and 9-ODA both attracted drones was surprising. Most, if not all, sex-pheromone studies so far have revealed two different types of male behavior; either males are attracted by the whole species-specific pheromone blend, or the detection of the major pheromone component is sufficient to elicit male search flights and mating. In the latter case, the minor pheromone components increase the behavioral response, but minor components are incapable of inducing mating flights (Linn et al. 1986).

There are two possible interpretations for our results. First, we cannot definitely exclude that the attraction of drones to 9-ODA might be an experimental artifact. 9-ODA is present in low amounts in the mandibular glands (Plettner et al. 1997; Keeling et al. 2000), whereas we used high dosages in our experiments. Thus, it is possible that off-range dosages of 9-ODA resulted in activation of 10-HDA sensitive olfactory sensory neurons or upstream neural circuits, which lead to an artificial activation of the behavioral response to 10-HDA. Unfortunately, nothing is known about the receptor and neural processing for 10-HDA and possible interactions with 9-ODA.

The other possible interpretation is that our findings demonstrate a species specific behavioral response of *A. florea* drones, namely that they are responding to both 10-HDA and 9-ODA when presented as single compounds. *A. florea* drones are unique in that they have a strongly reduced sex-pheromone olfactory subsystem compared to drones of other honey bee species (Brockmann and Brueckner 2001, 2003). For example, *A. florea* drones only have about 1,200 olfactory poreplate sensilla compared to 14,700 in *A. mellifera* drones (Brockmann and Brueckner 2003). These quantitative differences in the peripheral olfactory system are in part due to the considerable differences in body size among honey bee species. As there is a high selection pressure on the drones' capability to detect queens, the responses to both pheromone components might suggest that *A. florea* drones have evolved unique sensory and central neural mechanisms to overcome limitations of their body size.

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Attraction of New Zealand Flower Thrips, *Thrips obscuratus*, to *cis*-Jasmone, a Volatile Identified from Japanese Honeysuckle Flowers

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Abstract This work was undertaken to identify floral compound(s) produced by honeysuckle flowers, *Lonicera japonica* (Thunberg), that mediate the attraction of New Zealand flower thrips *Thrips obscuratus* (Crawford). Volatiles were collected during the day and night and analyzed by gas chromatography–mass spectrometry (GC-MS) to determine their emission over these two periods. Nine compounds were identified in the headspace; the main compound was linalool, and the other compounds were germacrene D, *E*,*E*-*alpha*-farnesene, nerolidol, *cis*-jasmone, *cis*-3-hexenyl acetate, hexyl acetate, *cis*-hexenyl tiglate, and indole. There was a quantitative difference between day and night volatiles, with *cis*-3-hexenyl acetate, hexyl acetate, *cis*-hexenyl tiglate, and *cis*-jasmone emitted in higher amounts during the day compared to the night. When the compounds were tested individually in field trapping experiments, only *cis*-jasmone attracted New Zealand flower thrips in a significant number. In another field trapping experiment, *cis*-jasmone caught similar numbers of New Zealand flower thrips compared to a floral blend formulated to mimic the ratios of the compounds emitted during the day, while catch with the night-emitted floral blend was not significantly different from the control. Subsequently, two field trapping experiments were conducted to determine the optimal attraction dose for *cis*-jasmone, a range of 1–100 mg loaded onto a red rubber stopper was tested, and the highest catches were in traps baited with 100 mg loading. A higher range of 100–1000 mg loaded into polyethylene vials was tested, and the highest catch was in traps baited with 500 mg. In another experiment aimed at comparing the attraction efficacy of *cis*-jasmone with the two other known thrips attractants (ethyl nicotinate and *p*-anisaldehyde), ethyl nicotinate showed the highest trap catch followed by *cis*-jasmone. A smaller number of *Thrips tabaci* (Lindeman) was attracted to traps baited with *cis*-jasmone. These results suggest that *cis*-jasmone might act as a kairomone that mediates the attraction of New Zealand flower thrips to the flowers of the Japanese honeysuckle.

Keywords *Thrips obscuratus* · *Thrips tabaci* · Japanese honeysuckle · *cis*-Jasmone · Ethyl nicotinate · Kairomone · Thysanoptera · Thripidae

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Introduction

Thrips generally are considered economically important pests in vegetable, fruit, floriculture, and plant production throughout the world. New Zealand flower thrips, *Thrips obscuratus* (Crawford) damages nectarines by feeding on fruitlets (McLaren 1992). Adult *T. obscuratus* also are attracted to ripening stone fruits, causing quarantine problems for export fruits (McLaren and Fraser 2002). Some thrips can transmit viral, fungal, and bacterial

pathogens to plants (Fermaud et al. 1994; Mound 2005). Insecticides are used to control thrips populations on nectarines (McLaren and Fraser 2000). The spray threshold is based on manual monitoring of thrips (McLaren and Fraser 2002). However, success with this technique is dependent on weather patterns, and it has associated sampling errors. Thus, the development of a practical synthetic attractant for *T. obscuratus* could help to determine accurately the level of thrips present and help reduce the use of insecticides or could be used directly in a control tactic, e.g., mass trapping or lure and kill (El-Sayed et al. 2006, 2009).

Many flower-dwelling thrips species feed on pollen and nectar of various plants, which suggests that they may play a role in the pollination of these species. The efficacy of thrips as pollinators has not been studied systematically. However, there is a tendency to consider thrips as pollen thieves rather than effective pollinators (Mound 2005). New Zealand flower thrips, *T. obscuratus*, has been found in the flowers, leaves, and fruits of many native and exotic plants in New Zealand (Norton 1984; Teulon and Penman 1990). It has been suggested that the attraction of thrips to host plants is regulated by both olfactory and visual cues (Teulon et al. 1999). Several plant volatile compounds attract different thrips species in the field (see El-Sayed 2008). For example, ethyl nicotinate attracts *T. obscuratus* in New Zealand (Penman et al. 1982; Teulon et al. 1993). *p*-Anisaldehyde is attractive to several species of flower-dwelling thrips in England (Kirk 1985) and *Frankliniella occidentalis* (Pergande) in New Zealand (Teulon et al. 1993). Methyl anthranilate attracts two species of flower-dwelling thrips species, *Thrips coloratus* (Schmutz) and *Thrips hawaiiensis* (Morgan) in Japan (Murai et al. 2000). Elucidation of the behavioral activity of these compounds has been based on field screening experiments of compounds, rather than on the classical experimental approach to identify behaviorally active compounds from natural sources, in this case flowers. Thus, the ecological basis for attraction of thrips to ethyl nicotinate or similar compounds is poorly understood.

The honeysuckles (family Caprifoliaceae) have pleasant-scented flowers on vines in the genus *Lonicera*. There are more than 190 species worldwide that are distributed mainly in the Northern Hemisphere. The volatile constituents of some species have been investigated due to their commercial potential in the perfume industry, e.g., *Lonicera periclymenum* L. (Lamparsky 1985), *Lonicera americana* (Koch) (Mookherjee et al. 1990), and *Lonicera japonica* (Thumb) (Schlotzhauer et al. 1996; Miyake et al. 1998). Although these studies showed quantitative and qualitative variation in volatiles among different species, the volatiles were represented mainly by linalool and linalool derivatives such as linalool oxides, aldehydes, *cis*-jasmone, *cis*-jasmone lactone, and sesquiterpenes. Various odor blends that were formulated based on the constituents of flowers of the Japanese honeysuckle were attractive to many lepidopteran species in field trapping experiments (Pair and Horvat 1997).

Our objective, within the context of applications of attractants for insect pest management and border biosecurity surveillance programs in New Zealand, is to develop environmentally friendly semiochemicals that can be used for monitoring and control of insect pests and new invasive species. Many thrips species are aggressively invasive and can cause serious damage in newly invaded habitats. Therefore, the development of attractants for thrips would allow for monitoring, detection, delimitation, and surveillance. We have observed the attraction of significant numbers of *T. obscuratus* to the flowers of the Japanese honeysuckle, although this plant is not native to New Zealand. This work was undertaken to identify the chemical signal. We report in this study the results of the chemical analysis of volatiles of the Japanese honeysuckle flowers that were attractive to New Zealand flower thrips. In addition, we report the results of several field trapping experiments aimed at the identification of compound(s) that mediate the attraction.

Materials and Methods

Chemicals Linalool (>95%), *cis*-3-hexenyl acetate (98%), hexyl acetate (99%), indole (99%), *cis*-hexenyl tiglate (97%), and nerolidol (98%) were obtained from Sigma-Aldrich Chemical Company (St Louis, MO, USA). *cis*-Jasmone (99.5%) was obtained from Bedoukian Research Inc. (Danbury, CT, USA). Ethyl nicotinate (>99%), *p*-anisaldehyde (98%), and germacrene D (60%) were obtained from Fluka, (Steinheim, Germany), Aldrich (Milwaukee, WI, USA), and R.C. Treatt and Co. Ltd (Suffolk, UK), respectively. *E,E-alpha*-farnesene (99%) was extracted from apples as described in Murray (1969). All chemicals were stored at −5°C until used.

Volatile Collection and Analysis Volatiles were collected from flowers in the field by using a dynamic headspace collection method followed by sorbent extraction. Collection was conducted under natural light conditions and ambient temperature and was conducted in two subsequent time periods to determine the emission during the day (0800–1900 hours) and night (1900 to 0800 hours). Intact flower heads with few leaves was housed in an oven bag (Pams oven bag, polyester, Auckland, New Zealand, i.d. 4.0 cm, 6.0 cm high). A charcoal-filtered air stream was pulled over the flowers, and the headspace was collected on an adsorbent trap containing 50 mg of Tenax-GR 35/60

(Alltech Associates Inc.) in a 15-mm long×10-mm diameter glass tube. Tenax traps were thermally conditioned at 200°C under a stream of nitrogen before use. Airflow in the headspace collection system was 2 l/min, and each collection session lasted for 24 h. Control samples were collected from the above system but without flowers to distinguish between floral compounds and ambient contaminants. After volatile collection, Tenax traps were transported to the lab and extracted with 1 ml of hexane (5×200 µl aliquots, *n*-hexane analaR BDH, Laboratory Supplies, Poole, England). Quantification of compounds was conducted by using external standard methods. Sample volumes were reduced to 10 µl at ambient temperature under a stream of argon. Samples were sealed and stored at −80°C until used. The concentrated extracts of headspace from flowers were analyzed with a Saturn 2200 GC-MS (Varian Inc., Walnut Creek, CA, USA). The GC-MS system was equipped with a 30 m×0.25 mm i.d.×0.25 µm, VF5-MS capillary column (Varian Inc.). Helium was used as carrier gas. Spectra were recorded at an ionization voltage of 70 eV over a mass range *m*/*z* of 20 to 499. The transfer line and the ion trap were held at 250°C and 180°C, respectively. Compounds were identified by comparing their mass spectra with the authentic standards and the NIST MS library, as well as coincidences for Kovats retention indices published in the literature (El-Sayed 2008). For analysis of liquid samples, the oven was programmed from 40°C (held for 2 min) to 240°C at 4°C/min. Samples were injected in splitless mode, and the temperature of the injector was maintained at 220°C.

Field Trapping Experiments

Testing Individual Compounds The relative attractiveness of the individual compounds identified in the headspace of (*cis*-3-hexenyl acetate, hexyl acetate, linalool, indole, *cis*-hexenyl tiglate, *cis*-jasmone, germacrene D, *E,E-alpha*-farnesene, and nerolidol) were tested in a peach orchard (Yumyeong) near Lincoln, Canterbury for attraction of *T. obscuratus* in January of 2006. Traps were deployed for 2 weeks. Ten milligrams of each compound dissolved in 200-µl hexane were loaded into the well of 8-mm diameter red rubber septa (Thomas Scientific Inc., Philadelphia, PA, USA), and the solvent was allowed to evaporate in a fume hood. Septa were stored at −20°C until use. Lures were replaced after 5 days around the middle of the test period. Red delta traps made of plastic corflute contained a white adhesive-coated panel (Suckling and Shaw 1992) baited with individual compounds and were placed in five rows with three replicates of each treatment. Red delta traps were used because they are less attractive to thrips, as well as non-target species including bees and bumble bees compared to white traps. Traps were positioned 1.7 m above the ground in each trap tree and were spaced 10 m apart in each row. Each treatment was assigned randomly to a trap tree within each row of trees. Septa were placed in the center of the white sticky base lying on the sticky surface. Sticky bases were removed weekly during the experimental period for later counting. Stereomicroscopy was used to aid the identification and counting of thrips caught on the sticky bases. A trap with a blank lure was used as control.

Testing blends of compounds In subsequent experiment, the relative attractiveness of two floral blends that reflected day and night floral compounds were formulated and tested for 2 weeks in January 2009 by using a randomized complete block design. Lures were replaced after 5 days around the middle of the test period. Based on the result of the first experiment, *cis*-jasmone was used as a positive control, and a trap baited a blank lure was used as a negative control. The loadings of two floral blends and *cis*-jasmone are given in Table 2. Floral odors tested were released using 12-mm diameter polyethylene vials (La Packaging, Yorba Linda, CA, USA). The polyethylene vials containing the odor blend were stored at −20°C until use. The experimental design and protocol were identical to the above experiment.

Dose–Response Experiments The effect of three doses of *cis*-jasmone (1, 10, and 100 mg) loaded into red rubber septa on the number of *T. obscuratus* captured in *cis*-jasmone-baited traps was assessed in a peach orchard (Yumyeong) near Lincoln, Canterbury, for 2 weeks in February 2006 by using a randomized complete block design. *cis*-Jasmone was loaded into red rubber septa. A septa was loaded with 1, 10, or 100 mg of *cis*-jasmone dissolved in 200 µl hexane, and the solvent was allowed to evaporate in a fume hood. The septa were stored at −20°C until use. Traps were placed in five rows with five replications for each treatment at 15 m spacing between treatment rows. Septa were placed in the center of the white sticky base lying on the sticky surface. One of each of the four treatments was randomly assigned to a trap tree within each row of trees. In a separate experiment, with Yumyeong peaches separated by shelter belts from the first dose–response test, the effects of higher doses of *cis*-jasmone (100, 500, and 1,000 mg) loaded into polyethylene vials were assessed for 2 weeks in February 2006. The three higher doses 100, 500, and 1000 mg of *cis*-jasmone were loaded directly into polyethylene vials. The experimental design and protocol in the second experiment were identical to that of the first dose–response experiment. In both experiments, a trap with a blank lure was used as control.

Table 1 Mean amounts (ng)± SE of volatiles identified in the headspace of the Japanese honeysuckle flowers, *Lonicera japonica*

Compound	[a]Kovats	Amount (ng)		*P* value
		Day	Night	
cis-3-hexenyl acetate	1006	6.8±1.13	2.3±0.38	0.01
Hexyl acetate	1011	2.6±0.49	1.2±0.27	0.04
Linalool	1103	108.6±23.8	101.4±18.34	0.81
Indole	1288	1.4±0.56	0.5±0.17	0.14
cis-3-hexenyl tiglate	1327	1.5±0.28	0.5±0.11	0.01
cis-Jasmone	1393	6.9±1.30	2.7±0.38	0.01
Germacrene D	1480	21.4±4.80	31.6±5.24	0.23
E,E-alpha-farnesene	1489	17.1±4.90	11.3±2.30	0.31
Nerolidol	1093	14.9±4.58	7.7±2.54	0.23

Compound identities were confirmed by comparison of MS and retention time of authentic standards and the published kovats value

[a] Kovats index on a DB-5 column

Relative Attractiveness of cis-Jasmone vs. Known Thrips Attractants The relative attractiveness of *cis*-jasmone, ethyl nicotinate, and *p*-anisaldehyde was tested in field trapping experiments in peach orchards for 2 weeks in early March 2006 in Canterbury. Five hundred milligrams each of *cis*-jasmone, ethyl nicotinate, and *p*-anisaldehyde were loaded directly into 12-mm-diameter polyethylene vials. A trap with a blank lure was used as a control. A randomized complete block experimental design was used, with a layout and experimental design similar to that described above for the dose response experiments.

Data Analysis

The variance of the mean quantity of each compound emitted by honeysuckle flowers during the day and night or the mean captures obtained with each treatment was stabilized by using the $\sqrt{x}$ or $\sqrt{(x+1)}$ transformation, respectively. The significance in the quantity of compounds emitted or the treatment effect in the field experiments were tested by using analysis of variance (SAS Institute Inc. 1998). Significantly different means were identified by using Fisher's protected least significant difference.

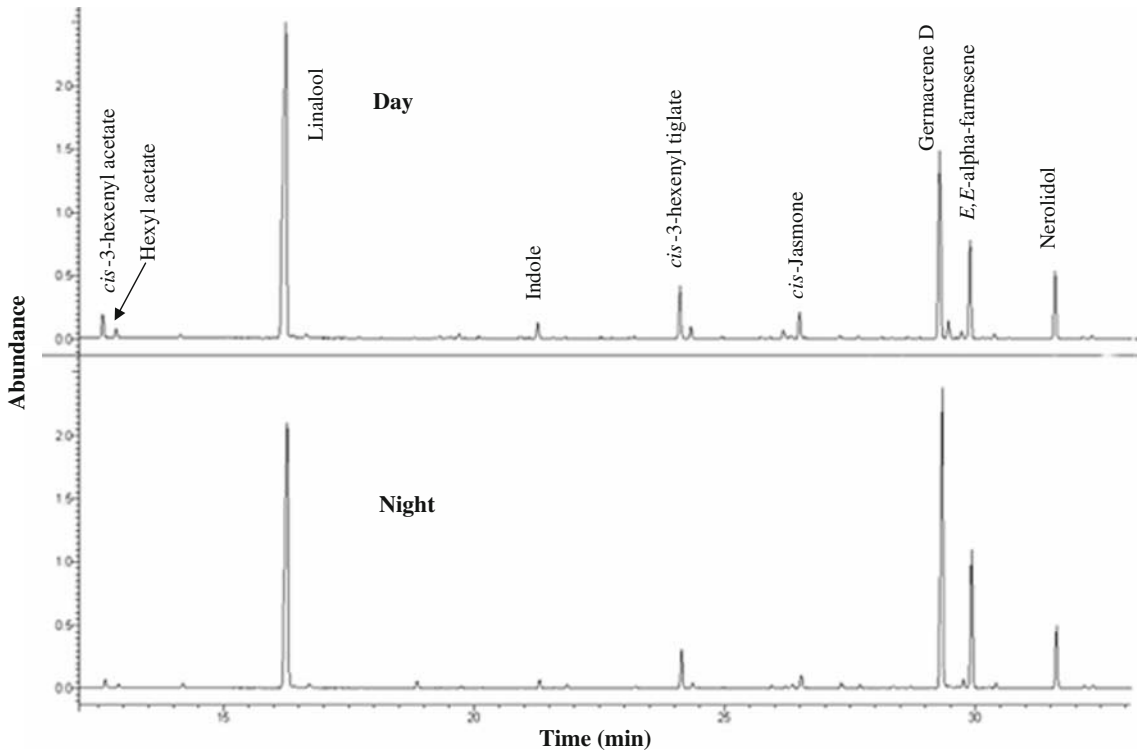

Fig. 1 GC traces of extracts of headspace volatile chemicals collected from of the Japanese honeysuckle flowers, *Lonicera japonica* during two periods (day and night)

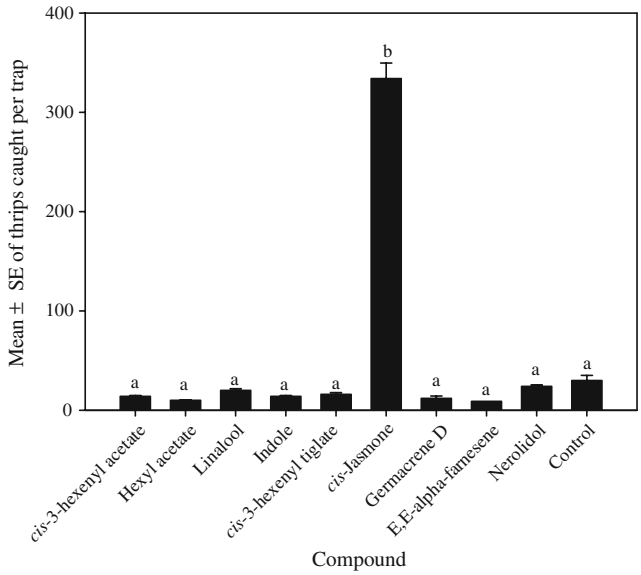

Fig. 2 Mean catch±SE of New Zealand flower thrips, *Thrips obscuratus* in traps baited with compounds (10 mg each) identified in the headspace of the Japanese honeysuckle flowers, *Lonicera japonica*. *Letters on columns* indicate significant differences ($P<0.05$)

Results

Headspace Chemical analyses of the headspace of the honeysuckle flowers from the dynamic headspace collection method are given in Table 1. Nine compounds were identified (Fig. 1) with the main compound, linalool, contributing up to 60% and 64% of total headspace volatiles emitted during the day and the night, respectively (Table 1). The other compounds were germacrene D, *E,E-alpha*-farnesene, nerolidol, *cis*-jasmone, *cis*-3-hexenyl acetate, hexyl acetate, *cis*-hexenyl tiglate, and indole. There was a quantitative difference between day and night volatiles, with *cis*-3-hexenyl acetate (treatment $F_{1,8}=14.22$, $P>0.01$), hexyl acetate (treatment $F_{1,8}=6.4$, $P>0.01$), *cis*-hexenyl tiglate (treatment $F_{1,8}=10.6$, $P>0.01$), and *cis*-jasmone (treatment $F_{1,8}=9.7$, $P>0.01$) emitted in higher amounts during the day.

Testing Individual Compounds and Blends of Compounds When the compounds were tested individually, only *cis*-jasmone attracted New Zealand flower thrips in significant numbers compared to control (treatment, $F_{9,20}=23.5$, $P<0.001$, Fig. 2). The catch in traps baited with floral blend volatile mimics emitted during the day and with *cis*-jasmone was significantly higher than control (Table 2). The catch in traps baited with day blend volatiles was similar to *cis*-jasmone alone. However, the catch in traps baited with blend volatiles emitted during the night was not different from the catch in control traps (Table 2).

Dose–Response Experiments The amount of *cis*-jasmone loaded onto red rubber septa affected the number of *T. obscuratus* captured in the two experiments (treatment, $F_{3,16}=15.1$, $P<0.05$ for the lower dose; treatment, $F_{3,16}=8.94$, $P<0.01$ for the higher dose). In the experiment, the greatest number of thrips was captured at the 100 mg loading (Fig. 3A). There was no difference in the mean numbers of thrips captured using the 0, 1, and 10 mg loading, while increasing the loading from 10 to 100 mg resulted in a significant increase in the mean number of *T. obscuratus* captured (Fig. 3A). In the second dose–response test, the greatest number of thrips captured was at the 500 mg loading (Fig. 3B). Increasing the loading from 100 to 1,000 mg resulted in a significant increase in the mean number of *T. obscuratus* (Fig. 3B).

Table 2 Mean±SD of catch of New Zealand flower thrips, *Thrips obscuratus* in traps baited with two floral odor blends represent odor emission in two periods (day and night) from the Japanese honeysuckle flowers, *Lonicera japonica*, and *cis*-jasmone

Chemical	Blend (mg)			Control
	Day	Night	One-component	
cis-3-hexenyl acetate	8	3	10	
Hexyl acetate	2	2		
Linalool	118	127		
Indole	2	1		
cis-3-hexenyl tiglate	2	1		
cis-Jasmone	10	2		
Germacrene D	24	40		
E,E-alpha-farnesene	18	14		
Nerolidol	16	10		
Mean catch of *T. obscuratus**	386 a	142 ab	412 a	105 b
±SE	55.6	7.2	84.5	23.1

Mean catch followed by the same letter are not significantly different ($P>0.05$)

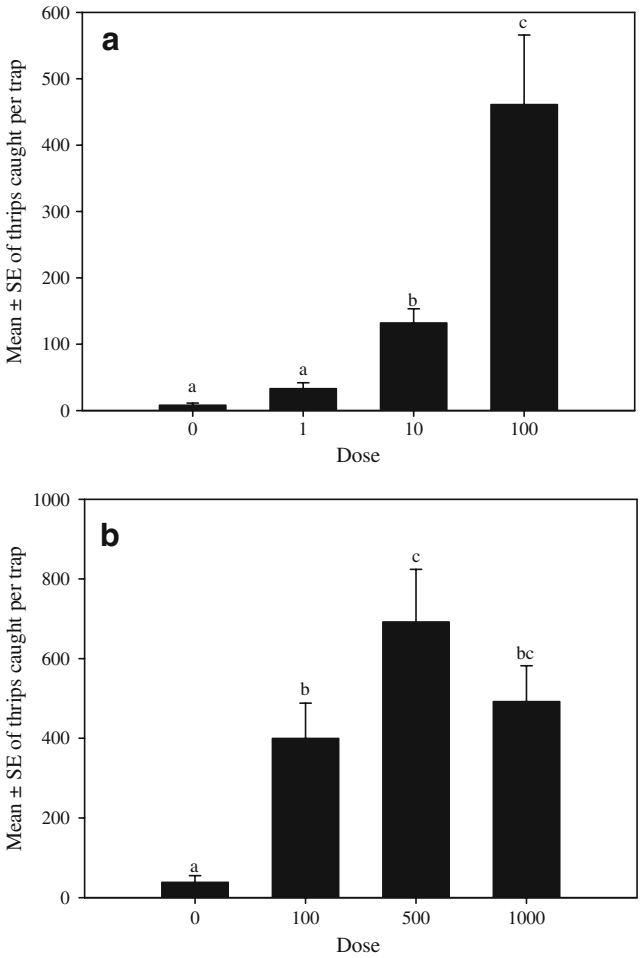

Fig. 3 Mean catch±SE of New Zealand flower thrips, *Thrips obscuratus* in red delta traps baited with various dosages of *cis*-jasmone: **A** 0, 1, 10, and 100 mg loaded into red rubber septa; **B** 0, 100, 500, and 1000 mg loaded into polyethylene vials. *Letters on columns* indicate significant differences ($P<0.05$)

Relative Attractiveness of cis-Jasmone vs. Known Attractants The relative attractiveness of *cis*-jasmone, ethyl nicotinate, and *p*-anisaldehyde to *T. obscuratus* is shown in Fig. 4. More thrips were caught in red delta traps baited with ethyl nicotinate than *cis*-jasmone or *p*-anisaldehyde (Treatment $F_{3,16}=24.6$, $P<0.01$). *Cis*-jasmone attracted more thrips than *p*-anisaldehyde and control traps. There was no significant difference between attraction to *p*-anisaldehyde and control trap.

Discussion

Although nine compounds were positively identified in the headspace of the honeysuckle flowers, other compounds were present in trace amounts. The number of compounds emitted from flowers was less than the compounds identified in the flower extracts (Schlotzhauer et al. 1996). Except for *cis*-3-hexenyl acetate, all compounds found in the headspace were present in the flower extracts (Schlotzhauer et al. 1996). There was similarity between the ratios of the compounds found in the flower headspace and the ratios found in steam distilled floral extracts. Linalool was the main compound in both the extracts and the headspace samples, while germacrene D was the second main component. Miyake et al. (1998) tentatively identified six compounds in the flower headspace of Japanese honeysuckle, with linalool as the main one and others including indole, germacrene D, nerolidol, *cis*-hexenyl tiglate, and decanal present in much lower amounts. *E,E-alpha*-farnesene was not reported, while it was present in our headspace samples. Miyake et al. (1998) showed that there is a rhythmic emission of volatiles from honeysuckle flowers where both linalool and germacrene D are emitted in large quantities around midnight. In contrast, in our analysis, there was no difference in the emission of linalool and germacrene D between the two collection periods. This difference could be due to the sampling method; we estimated the emission during 12-h periods, while they sampled at 3-h intervals.

When the compounds in the headspace were tested individually, only *cis*-jasmone attracted thrips in significant numbers. A floral blend formulated to mimic the odor produced by honeysuckle flowers during the day attracted similar numbers of thrips as *cis*-jasmone alone, while the attraction to the floral blend formulated to mimic the odor produced by flowers during the night was similar to the control. This suggests that *cis*-jasmone is the only active compound in the floral blends tested. Mookherjee et al.

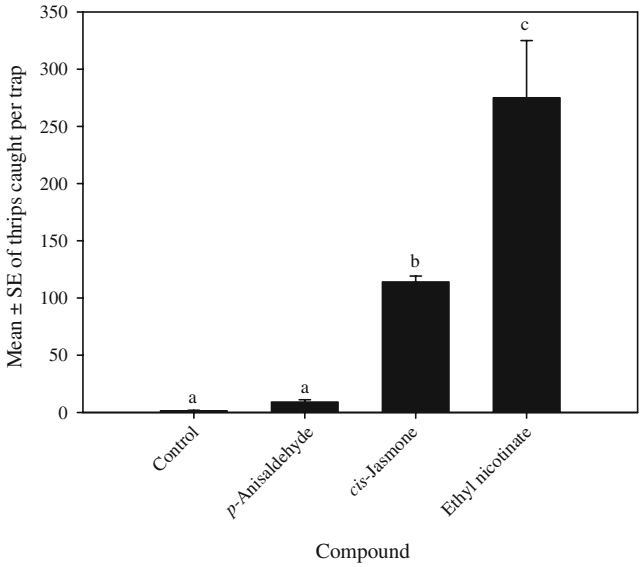

Fig. 4 Mean catch±SE of New Zealand flower thrips, *Thrips obscuratus* in red delta traps baited with 500 mg of *cis*-jasmone, ethyl nicotinate, and *p*-anisaldehyde. *Letters on columns* indicates difference ($P<0.05$)

(1990) showed that both *cis*-jasmone and indole levels maximize during the day in the flower of *L. americana*. In our analysis, *cis*-3-hexenyl acetate, hexyl acetate, *cis*-3-hexenyl tiglate, and *cis*-jasmone levels all maximized during the day with the latter likely attracting the diurnally active New Zealand flower thrips (McLaren and Fraser 2002).

cis-Jasmone attracted New Zealand flower thrips in the field, and its attraction can be considered moderate compared to ethyl nicotinate. Ethyl nicotinate was reported as a potent attractant for New Zealand flower thrips by Penman et al. (1982). *p*-Anisaldehyde, another known thrips attractant (Kirk 1985), was not attractive to *T. obscuratus* in our study. In contrast, *p*-anisaldehyde was attractive to the Western flower thrips, *F. occidentalis* (Kirk 1985; Teulon et al. 1993). The behavioral basis of attraction of New Zealand flower thrips to ethyl nicotinate is not clearly understood and needs to be resolved. It is known that both olfactory and visual cues are important components in host-finding behavior of New Zealand flower thrips (Teulon et al. 1993), and it is likely that both senses are used for host location. *T. obscuratus* is an indigenous species of New Zealand and has a wide host range (Norton 1984; Teulon and Penman 1990), being attracted to flowers and ripe stone fruits such as peaches, which suggest that odors are an important cue for host location. Thus, *cis*-jasmone might act as a kairomone used by thrips as a foraging cue.

cis-Jasmone is produced by damaged plants, and there is evidence that it plays a role in plant defense against herbivores (Birkett et al. 2000; Bruce et al. 2003). It was found repellent to the lettuce aphid, *Nasonovia ribisnigri* (Mosh) in olfactometer bioassays and to the damson-hop aphid, *Phorodon humuli* (Schrank) in the field (Birkett et al. 2000). It was found also to be repellent to the alatae of the grain aphid, *Sitobion avenae* (Fabricius) in an olfactometer bioassay (Bruce et al. 2003). Application of *cis*-jasmone on wheat reduced the population of cereal and grain aphids, *S. avenae*, and the plant was less susceptible to attack by aphids (Birkett et al. 2000 and Bruce et al. 2003). Additionally, *cis*-jasmone was attractive to the predacious seven-spot ladybird, *Coccinella septempunctata* L., in an olfactometer, and to the aphid parasitoid, *Aphidius ervi* (Haliday), in wind tunnel studies (Birkett et al. 2000). *cis*-Jasmone also was attractive to the Japanese beetle, *Popillia japonica* (Newman) in field trapping experiments (Loughrin et al. 1998). It seems unlikely that the attraction of New Zealand flower thrips, *T. obscuratus* to *cis*-jasmone is related to plant defense mechanisms, as in this case, *cis*-jasmone is released by undamaged flowers, whereas in other plant species such as cotton or potato, it is released more after damage.

cis-Jasmone has been reported in the floral odor of many plant species (see http://www.pherobase.com/database/floral-compounds/floral-taxa-compounds-detail-cis-jasmone.php). Examples include the kiwifruit flower, *Actinidia chinensis* (Planch.) (Tatsuka et al. 1990), which is usually visited by *T. obscuratus* and leads to infection by the botrytis bunch rot, *Botrytis cinerea* (Fermaud et al. 1994). *cis*-Jasmone also has been found in the flowers of *Nicotiana tabacum* (L.) (Solanaceae) (Loughrin et al. 1990). The onion thrips, *Thrips tabaci* (Lindeman) is a cosmopolitan species and is known to attack a wide range of host plants including tobacco *N. tabacum*. Interestingly, we have caught a smaller number of *T. tabaci* in traps baited with *cis*-jasmone in the peach orchard. The occurrence of *cis*-jasmone in the flowers of *N. tabacum* and the attraction of *T. tabaci* to *cis*-jasmone might support our view on the behavioral mechanism that underlies attraction of thrips to *cis*-jasmone as a response to host volatile compounds. The finding that it attracted *T. tabaci* in a peach orchard suggests the need for further investigation of the attraction of *cis*-jasmone to *T. tabaci* in regard to host plant selection (i.e., onion, tomatoes, and tobacco). Although *cis*-jasmone has been identified in the headspace of flowers of several plants, we did not find evidence in the literature to indicate that this compound attracts pollinator insects.

Both odor and color play a role in attraction of thrips to host plants (Teulon et al. 1999), and New Zealand flower thrips show a preference for white over any other color (McLaren 1995). It would be interesting to study the interaction between different colors and *cis*-jasmone in the attraction of *T. obscuratus*. *cis*-Jasmone and ethyl nicotinate have attracted *T. obscuratus*, so it would also be interesting to study the interaction between the two compounds in a binary blend. Comparing the potency of both compounds on thrips attraction over the entire flight season would also be valuable. Although *cis*-jasmone showed moderate activity compared to ethyl nicotinate, it is more environmentally friendly and less hazardous to human and animal health than ethyl nicotinate, which has hazardous properties (Fisher Scientific Inc. 2007). Our researchers have to wear masks during the deployment of ethyl nicotinate lures. This advantage makes a *cis*-jasmone lure more desirable for *T. obscuratus* population monitoring.

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Performance and Secondary Chemistry of Two Hybrid Aspen (*Populus tremula* L. x *Populus tremuloides* Michx.) Clones in Long-Term Elevated Ozone Exposure

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Abstract The effects of moderately elevated ozone (ca. 35 ppb) on the growth and secondary chemistry of the leaves of two soil-grown Finnish hybrid aspen (*Populus tremula* L. x *Populus tremuloides* Michx.) clones with different ozone sensitivities were studied at an open-air exposure field in Kuopio, Finland. Stomatal conductance, photosynthetic rate, and chlorophyll fluorescence were measured during the third growing season. Foliar phenolic concentrations, ergosterol concentration of fine roots, and final dry mass of the trees were determined at the end of the third growing season. Elevated ozone increased the ectomycorrhizal status of the fine roots but had no effect on gas exchange or on the final biomass of either of the clones, indicating equal sensitivity to ozone and no effect of elevated ozone on the intraspecific competitive ability of the clones after three growing seasons. However, in agreement with the data from potted plants of the same clones after two growing seasons, significant differences between the clones were found in all parameters measured. A negative correlation between growth and high concentrations of foliar phenolics indicated that allocation to secondary chemistry also was costly in terms of growth under high resource availability.

Keywords Hybrid aspen · Clones · Ozone · Sensitivity · Photosynthesis · Stomatal conductance · Phenolics · Flavonol glycosides · Salicylates · Condensed tannins · Competition · Ectomycorrhiza

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Introduction

Populus species are fast-growing pioneer trees of the temperate forests and are used in the wood industry for the production of paper, plywood, and matches (Zsuffa et al. 1996). They are also good candidates for biomass production for energy, as well as for phytoremediation of heavy metal-polluted soils (Hermle et al. 2007; Christersson 2008). Hybrid aspen, which is a cross between European aspen (*Populus tremula* L.) and North American trembling aspen (*Populus tremuloides* Michx.), has shown superior growth and wood quality compared with native European aspen (Yu et al. 2001). Plantations of selected hybrid aspen clones have been established in Sweden, Estonia, and Finland for pulpwood and bioenergy production

(Rytter and Stener 2005; Tullus et al. 2007; Luoranen et al. 2006 and references therein). However, there is large clonal variation in hybrid aspen in response to environmental conditions (Oksanen et al. 2001; Yu and Pulkkinen 2003; Häikiö et al. 2007, 2008). In a changing climate, it is important to find stress-tolerant genotypes for large-scale plantations.

Ground-level atmospheric ozone (O_3) remains a global air pollution problem despite precursor (NOx) emission reduction measures in industrialized countries during the last decades. Despite the reductions in short-term peak levels of ozone, background ozone concentrations have increased by 2 ppb per decade to the present concentrations of 35–40 ppb in the Northern Hemisphere (The Royal Society 2008). *Populus* species have been reported to be one of the most sensitive species to elevated tropospheric ozone concentrations (Wittig et al. 2007). Significant reductions in total biomass were observed in a trembling aspen stand after 7 years of exposure to elevated ozone (King et al. 2005). We previously studied the responses of eight hybrid aspen clones and two native aspen clones in an open-field experiment with moderately elevated ozone concentrations (ca. 35 ppb). We found a significant genotype x ozone interaction in the performance of potted saplings of hybrid aspen after two growing seasons, with elevated ozone reducing the growth of four of the clones and having no effect or enhancing the growth of the other four clones after two growing seasons (Häikiö et al. 2007).

In this study, parallel to an experiment with potted saplings of the same hybrid aspen clones, saplings of one moderately ozone-tolerant and one moderately ozone-sensitive clone (Oksanen et al. 2001; Häikiö et al. 2007) were planted in soil to study the effects of elevated ozone concentrations on their competitive ability. Exposure to elevated ozone concentrations was reported to cause changes in the intraspecific competitive ability of sensitive or tolerant aspen genotypes, with poor competitors having an added disadvantage under elevated ozone (Kubiske et al. 2007; Zak et al. 2007). We hypothesized that belowground competition together with long-term elevated ozone exposure would increase the competitive ability of the more ozone-tolerant clone relative to the more ozone-sensitive clone. We also hypothesized that the higher resource (nutrient) availability of the soil-grown trees would result in lower foliar phenolic concentrations compared with the potted trees, as reported by Kleiner et al. (1998) and Osier and Lindroth (2001). Phenolic concentrations were unaffected by nitrogen fertilization in our earlier study with potted hybrid aspen saplings (Häikiö et al. 2008). We also wanted to study the differences between the clones in the foliar phenolic composition observed earlier (Häikiö et al. 2008) in more detail.

Methods and Materials

Plant Material The material of the experiment consisted of two hybrid aspen (*Populus tremula x P. tremuloides*) clones included in the Finnish hybrid aspen breeding programme. Clones 55 and 110 were produced originally in 1952 and 1962, respectively, by crossing female *P. tremula* from Southern Finland with male *P. tremuloides* from Canada, and selected in 1998 from progeny trials in Anjalankoski (60°45′ N, 26°59′ E) and Lapinjärvi (60°37′ N, 26°11′ E), respectively. The clones were produced by micropropagation at the Finnish Forest Research Institute Haapastensyrjä Breeding Station and planted in soil as 1-yr-old saplings (30–40 cm in height) in June–July 2002 on an open-air exposure field at Kuopio University Research Garden (62° 53′ N, 27°37′ E). The soil was a former agricultural field and it was tilled, fertilized, and mixed with forest peat and sand before planting. Twenty four plants per clone were planted with 50 cm spacing on each of four replicate ozone exposure plots and four control plots. Saplings were grown under a natural microclimate without watering or fertilization except for one nitrogen fertilization in 2003 to level off differences in soil nitrogen status among the plots. Soil water content during the growing season was measured with a ThetaProbe ML2 (Delta-T Devices Ltd., Cambridge, UK). The volumetric soil water content varied between 0.30 m^3m^{-3} and 0.40 m^3m^{-3} during the growing season 2004 (data not shown). The biggest broad-leaved weeds were removed once in a growing season. For all samplings and measurements, first-flush leaves from primordia formed during the previous growing season were used.

Ozone Exposure A free-air ozone exposure system with four elevated ozone plots and four control plots was used to manipulate experimentally the ozone concentrations in the field. Ozone was produced from pure oxygen by an ozone generator (G21, Pacific Zone Technology Inc., Brentwood, CA, USA). It was released into exposure rings through vertical vent pipes from the upwind direction. Ozone fumigation was run 24 h day^{-1} in 2002–2003, and between 08:00–22:00 in 2004, and it was turned off during heavy rain, dew, low or high wind velocities, and when the ambient ozone concentration was less than 10 ppb. The fumigation was run from bud opening in May until leaf fall in October. Ozone concentrations were monitored at the canopy level in the center of the ozone exposure rings with two Dasibi 1008-RS ozone analyzers (Dasibi Environmental Corp., Glendale, CA, USA) and on the control rings with an Environnement S.A. O342M ozone analyzer (Environnement S.A., Poissy, France), which were intercalibrated. The realized ozone enhancement during the growing seasons 2002–2004 was 1.3–1.4 times the ambient

ozone concentration (approximately 35 ppb). Cumulative ozone exposures AOT00 and AOT40, together with the mean monthly temperatures for the growing seasons in 2002–2004, are given in Table 1. The mean daily ozone concentrations in 2002–2004 are presented in Fig. 1.

Leaf Nitrogen Concentration Samples for total leaf organic nitrogen concentration were collected in mid-June, 2004, and analyzed by the standard Kjeldahl method (Allen 1989). From each plot, 2 leaves from 5 trees per clone were taken and pooled for analysis, i.e., one sample per clone per plot (altogether 16 samples).

Chlorophyll Fluorescence, Stomatal Conductance, and Net Photosynthesis One sun leaf from 4 trees per clone per plot (altogether 64 leaves) in the middle of the canopy was selected and used in all fluorescence, conductance, and photosynthesis measurements. Measurements were made in dry, sunny weather between 10:00 and 16:00 h.

Chlorophyll fluorescence was measured four times in 2004 (June 23, July 12, July 27, and August 5) with a portable pulse-modulated FMS2 fluorometer (Hansatech Instruments Ltd., Norfolk, UK). After a 30-min dark adaptation, dark-adapted minimal fluorescence (F_0) was measured. A saturating pulse (0.7 s) of light (about 11 000 μmol m^{-2} s^{-1}) was applied, and maximal photochemical efficiency of PSII (F_v/F_m, where F_m is dark-adapted maximal fluorescence and F_v is F_m–F_0), was recorded.

Stomatal conductance (g_s) was measured four times in 2004 (June 17, July 19, July 22, and August 3) with LI-1600 Steady State Porometer (LI-COR Inc., NE, USA).

Leaf-level net photosynthesis (A_{sat}) was measured 5 times during the growing season 2004 (July 2, July 8, July 21, August 4, and August 17) using a CI-510 Portable Photosynthesis System (CID Inc., Vancouver, WA, USA). Photosynthesis was measured in saturating light (>1 000 μmol m^{-2} $s^{-}1$) after determining the light-saturation point of photosynthesis. In the last three measurements, a light attachment module (CI-510LA) was used because of unfavorable weather conditions.

Foliar Phenolics Samples for phenolic analyses were collected on August 4–9, 2004. Three leaves were taken and pooled from 3 trees per clone per plot (altogether 48 samples). The leaves were dried at room temperature with

Table 1 Ozone exposure and mean monthly temperatures during the growing seasons 2002–2004

		2002	2003	2004
AOT00 (ppm.h)	ambient O_3	60.8	76.5	46.3
	elevated O_3	77.4	116.3	65.4
AOT40 (ppm.h)	ambient O_3	0.5	1.3	0.9
	elevated O_3	16.6	17.7	10.4
mean O_3 exposure (ppb)	ambient O_3	36		
	elevated O_3	26		
mean temperature (°C)	May	–	12.3	9.0
	June	16.7	12.4	13.1
	July	19.2	20.2	16.9
	August	17.4	13.8	15.5
	September	10.0	10.2	11.5

Ozone exposure is expressed as accumulated over threshold of 0 ppb (AOT00) or 40 ppb (AOT40) for 24 h day^{-1}. Mean monthly temperatures were calculated from 24 h daily mean values

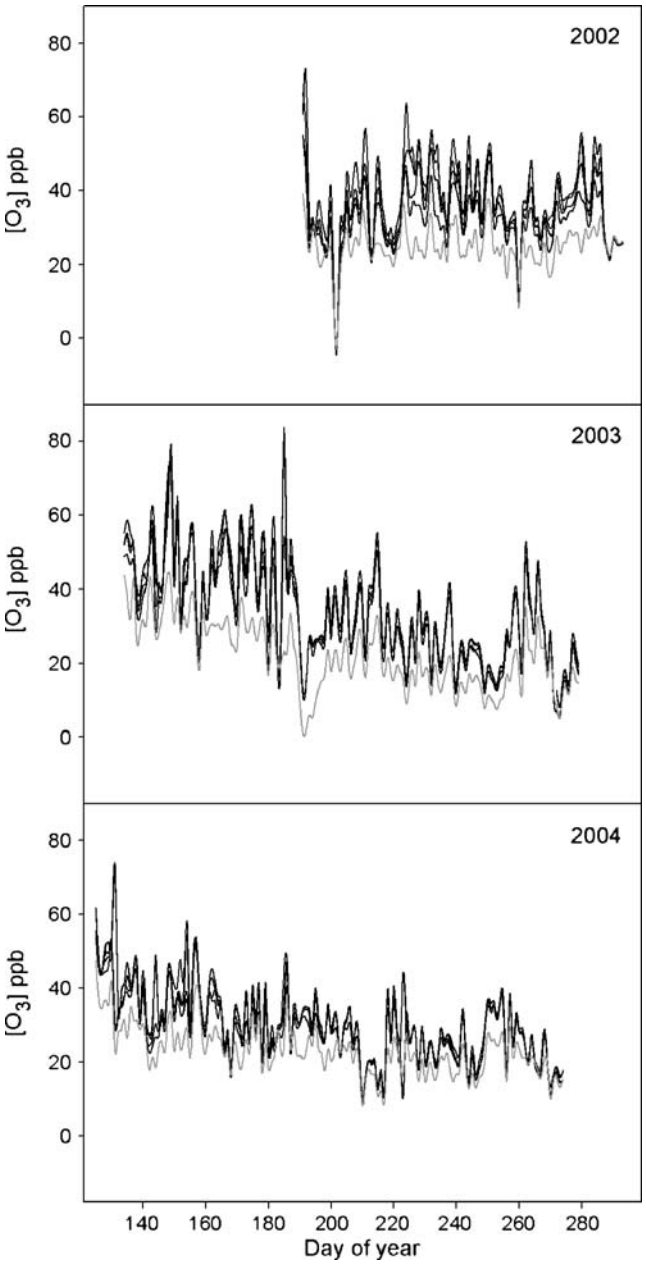


Fig. 1 Daily mean ozone concentrations (ppb) for 24 h day^{-1} in 2002–2004. *Black lines* = elevated ozone, *gray line* = ambient ozone

RH < 20%, and were ground to powder without the petiole and midvein. Dry samples were stored at −20°C until analyses. For extraction, 8 mg of leaf sample were mixed with 600 ml of ice-cold methanol. The sample was vortexed, left to stand on ice for 15 min, and centrifuged (3 min, 13 000 rpm). The supernatant was collected, and the residue was extracted another four times. Supernatants were combined, the methanol was evaporated under nitrogen flow, and the dry sample was stored at −20°C.

For HPLC analysis, the sample was redissolved in 600 ml methanol:H_2O (1:1). Phenolics were analyzed by using HPLC (HP 1050 Series HPLC, Palo Alto, CA, USA) with a binary pump, an autosampler, and a photo diode array detector, combined with ChemStation for LC 3D (Rev. A.09.01; Agilent Technologies). A 3 µm HP Hypersil ODS column (60×4.6 mm ID) was used. Eluents were aqueous 1.5% tetrahydrofuran + 0.25% *o*-phosphoric acid (=A) and 100% methanol (=B). The following gradient was used: 0–5 min: 100% A, 5–10 min 85% A, 10–20 min 70% A, 20–47 min 50% A, 47–65 min 100% B. The flow rate was 2 ml min^{-1}, and the injection volume was 15 µl. HPLC runs were monitored at 220 and 320 nm. HPLC-chromatograms from the HPLC analysis showed the main peaks at 220 nm (salicylates and catechins) and 320 nm (flavonoids) (Fig. 2). Initial identification of compounds was based on comparisons of retention times and spectral characteristics. Commercial standards were used for quantification of the identified compounds: (+)-catechin (Aldrich Chemicals Co, Milwaukee, WI, USA) for (+)-catechin and gallocatechin; chlorogenic acid (Aldrich) for neochlorogenic acid and chlorogenic acid; kaempferol 3-glucoside (Extrasynthèse, Genay, France) for kaempferol derivatives; quercetin 3-galactoside (Apin Chemicals Ltd, Abingdon, UK) for quercetin derivatives; myricetin 3-rhamnoside (Apin Chemicals Ltd.) for myricetin derivatives, isorhamnetin 3-glucoside (Apin Chemicals Ltd.) for the isorhamnetin derivative; salicin (Sigma, Steinheim, Germany); and tremulacin (Roth, Karlsruhe, Germany). Salicortin, disalicortin, and tremuloidin were based on purified standards obtained from prof. Beat Meier, ETH, Zürich, Switzerland (salicortin was used as a standard for both salicortin and disalicortin). Soluble condensed tannins were determined from an aliquot of the HPLC extract using the acid-butanol test (Porter et al. 1986). Tannin purified from hybrid aspen leaves was used as a standard. The mean value of three samples per clone per plot was used as a statistical unit.

For verifying the identification of compounds, one sample of each clone was analyzed by HPLC/API-ES mass spectrometry (HP 1100 Series LC/MSD, Hewlett-Packard, Palo Alto, CA, USA). A 3 µm HP Hypersil ODS column (100×2.1 mm ID) was used. Eluents were aqueous 1.5% tetrahydrofuran + 0.25% acetic acid (=A) and 100% methanol (=B). The following gradient was used: 0–5 min: 100% A, 5–10 min: 85% A, 10–20 min: 70% A, 20–60 min: 50% A, 60–62 min: 100% B. The injection volume was 5 µl, and flow rate was 0.4 ml min^{-1}. For electrospray ionization, the capillary was set at 4000 V, the nebulizer pressure was 30 psig, the drying gas temperature was 350°C with a flow of 12 l min^{-1}, and the fragmentation voltage was set at 80 V. Four novel phenolics were indentified: quercetin 3-arabinoglucoside (619 M+23; 465, M+1, quercetin-glucoside; 303, M+1, quercetin), quercetin 3-arabinoglucuronide (633, M+23; 479, M+1, quercetin-glucuronide; 303, M+1, quercetin), kaempferol 3-arabinoglucoside (603, M+23; 287, M+1, kaempferol), and isorhamnetin 3-glucuronide (493, M+21; 515, M+23; 317, M+1, isorhamnetin).

Biomass Analysis Twelve plants per clone per plot were harvested during August 20–24, 2004. Trees were measured for height, base diameter, and diameter at breast height (1.3 m). Plant parts were separated into leaves, stems (with branches), and roots, and the roots were washed. Only the main part of the coarse roots was collected from 3 trees per clone per plot. Plant parts were dried to constant weight at 60°C, and the dry weights of leaves, stems, and coarse roots were measured. Total leaf area was calculated from the dry weight of the leaves, after determination of dry weight/area ratio for each clone from sample leaves (3 leaves per clone per plot). The mean value of 12 plants per clone on each plot was used as a statistical unit, except for coarse roots, where the mean value of 3 plants per clone per plot was used. In addition, samples were collected to determine differences in fine root biomass between ozone and control plots since it was not possible to separate the fine roots of the two clones. Four samples per plot with a diameter of 5.3 cm and depth of 15 cm were cored in the middle of the plots between the trees in September 8, 2004. The samples were stored at −20°C until washed. Roots were separated into fine roots (< 2 mm diam) and coarse roots, dried to constant weight, and the dry weight was measured. The mean value of 4 samples per plot was used as a statistical unit.

Mycorrhizal Root Status The mycorrhizal status of the fine roots was determined by ergosterol analysis as described in Salmanowicz and Nylund (1988) and later modified by Nylund and Wallander (1992) and Markkola (1996). Briefly, four samples per plot with a diameter of 5.3 cm and depth of 11 cm were cored from the soil in the middle of the trees between August 26 and September 9, 2004. The fine roots were washed immediately after coring and stored at −80°C until analyses. The frozen fine roots were vacuum-dried and ground to powder in liquid nitrogen. Twenty five to 50 mg of the sample were mixed with 10 ml of 95% ethanol. The sample was shaken for 2 min, and 2 ml of 60% KOH was added for saponification. After incubation in a 95°C water bath for 45 min, the supernatant

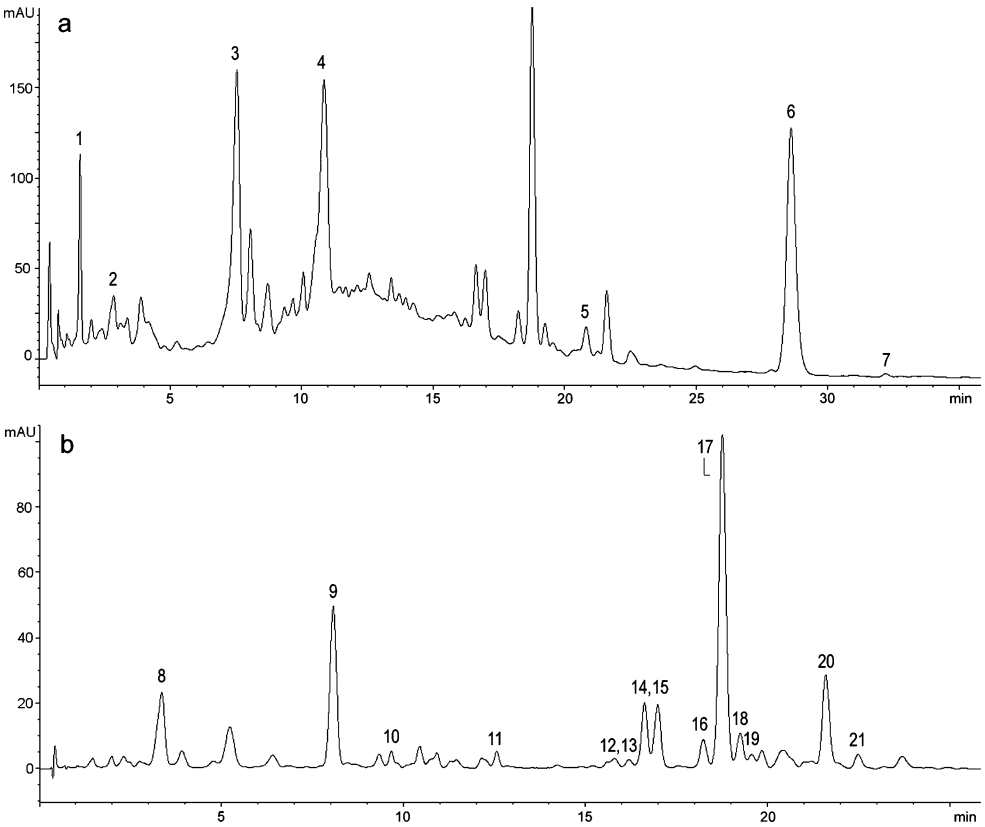


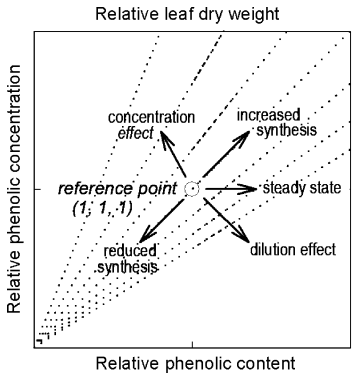
Fig. 2 HPLC chromatograms of identified hybrid aspen leaf phenolics at (**a**) 220 nm and (**b**) 320 nm. For identification of peaks, see Table 3

was decanted into a new tube with 3 ml of deionized water. The free ergosterol in the precipitate was extracted with 3, 2, and 1 ml pentane, and the pentane phases with the free sterols were collected into a new tube. Pentane was allowed to evaporate to dryness, and the dry samples were stored at −20°C. For HPLC analysis, ergosterol samples were dissolved in 500 µl of 100% methanol, and incubated at 50°C for 20 min. After centrifugation, the supernatant was transferred into the autosample vial. HPLC analysis was done by using a reverse-phase column (Hewlett-Packard, LiChrospher 100 RP-18) with 100% methanol as eluent. Twenty µl sample were injected and run at 1.6 ml per minute. Provitamin D2 (5,7,22-ergostatrien-3β-ol) was used as a standard.

Statistical and Graphical Analyses In the statistical analyses, the main effects and interactions of two levels of ozone and two clones were tested. In all analyses, the mean value per clone per plot was used as a statistical unit. To test for main effects and interactions of ozone and clone on biomass and growth, and on concentrations of phenolic compounds of leaves, linear mixed model analysis of variance using SPSS for Windows 14.0 (SPSS Inc. 2005) was performed, with ozone and clone as fixed factors, and plot as a random factor. For determinations of fine root biomass and ergosterol concentration, ozone was used as a fixed factor and plot as a random factor since clonal effects could not be studied. In the repeated measurements analyses (fluorescence, stomatal conductance, and photosynthesis), ozone, clone, and measuring date were used as fixed factors, and plot as a random factor. In the case of statistically significant interactions, pairwise *post hoc* comparisons were used for interpretation of the effects of different factors. For all statistical tests, the significance level was set at $P<0.05$.

Treatment and clone effects on foliar phenolics were analyzed also by using a graphical vector analysis (GVA; Haase and Rose 1995) to determine whether the induced/reduced accumulation of a compound is the result of increased/decreased synthesis, or concentration/dilution of the compound due to a change in leaf biomass (Koricheva 1999). Relative values of mean concentrations were plotted against mean contents per leaf, with the diagonal lines representing the relative mean foliar dry weights. Vectors were drawn by using either the ambient ozone treatment or clone 110 as the reference point (1, 1, 1), and the direction and magnitude of the vectors were used to interpret the impacts of ozone or clone on the phenolic compounds, respectively (Fig. 3).

Correlations between the two hybrid aspen clones, foliar phenolic compounds, and growth-associated variables were studied with principal component analysis (PCA) using SIMCA-P 11.5 software (Umetrics AB, Umeå, Sweden). After unit variance scaling of the variables, a model with four principal components that explained 89% of the variation was extracted. The input data matrix consisted



Content	Concentration	Leaf dry weight	Interpretation
+	0	+	Steady state (increase)
+ / 0	-	+	Dilution effect
-	-	+ / 0 / -	Reduced synthesis
-	0	-	Steady state (decrease)
0 / -	+	-	Concentration effect
+	+	+ / 0 / -	Increased synthesis

Fig. 3 Interpretation of directional shifts in the phenolic content (*x* axis), concentration (*y* axis) and leaf dry weight (*diagonal lines*) in a graphical vector analysis. Adapted from Haase and Rose (1995) Koricheva (1999) and Peltonen et al. (2005)

of 16 observations (2 clones × 8 plots) and 26 variables (22 phenolic compounds, stem and root dry weight, height growth, radial growth, and A_{sat}, F_v/F_m, and g_s measured in the beginning of August).

Results

Leaf Nitrogen Concentration Leaf nitrogen concentration was on average 32.6 mg g^{-1}. There were no statistically significant differences between the clones or between ozone treatments (data not shown).

Chlorophyll Fluorescence, Stomatal Conductance and Photosynthetic Rate Ozone had no main effect on F_v/F_m, g_s or A_{sat} of the clones (Table 2, Fig. 4). However, there was a significant O_3 x date interaction in A_{sat} showing higher photosynthetic rates in response to elevated ozone in the first measurement in July (P=0.026), but no differences between the ozone treatments in the rest of the measurements (Fig. 4). There were statistically significant differences between the clones in F_v/F_m, and A_{sat} but not in g_s (Table 2), with higher values in clone 110. There was a significant clone x date interaction in F_v/F_m, with clone 110 having higher F_v/F_m values than clone 55 except at the first measuring date (Table 2, Fig. 4).

Table 2 Main effects and interactions of ozone, clone, and measuring date on leaf-level net photosynthesis (A_{sat}), stomatal conductance (g_s), and maximum efficiency of PS II (F_v/F_m) of two clones of soil-grown hybrid aspen saplings

	df	A_{sat}	*df*	g_s	*df*	F_v/F_m
Ozone	1	0.676	1	0.889	1	0.150
Clone	1	**<0.001**	1	0.953	1	**<0.001**
Date	4	**<0.001**	3	**<0.001**	3	**<0.001**
Ozone * clone	1	0.371	1	0.646	1	0.073
Ozone * date	4	**0.029**	3	0.800	3	0.561
Clone * date	4	0.391	3	0.063	3	**0.041**
Ozone * clone * date	4	0.705	3	0.790	3	0.986

The *P* values are from linear mixed model ANOVA

Foliar Phenolics We extracted and analyzed, in addition to condensed tannins, 21 individual phenolic compounds from hybrid aspen leaves. Among them, four compounds, quercetin 3-arabinoglucoside, quercetin 3-arabinoglucuronide, kaempferol 3-arabinoglucoside, and isorhamnetin 3-glucuronide, were tentatively identified for hybrid aspen. Elevated ozone affected significantly only the concentrations of neochlorogenic acid, which were 57% and 28% higher in elevated ozone in clones 110 and 55, respectively (Table 3). Instead, there were statistically significant differences between the clones in all phenolic compounds, except for salicin and quercetin 3-arabinoside (Table 3). In total, clone 55 contained 83% higher concentrations of phenolics than clone 110. Of the main phenolic groups, salicylates comprised 45% and 57% of the total phenolics in clones 55 and 110, whereas condensed tannins and catechins comprised 46% and 29% of the total phenolics of clones 55 and 110, respectively. Phenolic acids together with flavonol glycosides comprised less than 14% of the total phenolics. Concentrations of most individual phenolic compounds were higher in clone 55, and only disalicortin, neochlorogenic acid, kaempferol diglycoside, kaempferol 3-glucuronide, and myricetin 3-glucoside + glucuronide were found in higher concentrations in clone 110 than in clone 55 (Table 3).

Vector analysis showed that elevated ozone resulted in increased accumulation of most phenolics, as shown in Table 3, but since the effects were not statistically significant, except for neochlorogenic acid, the results are not shown. Dry weights of single leaves were not different between the clones (0.11 g for clone 55 and 0.13 g for clone 110; P=0.209). Thus, when clone 55 was compared to clone 110 (Fig. 5 a–g), vector analysis showed that the higher concentrations of total phenolics, salicylates, and especially tannins and catechins in clone 55 were the result of increased synthesis and not a concentration effect (Fig. 5 a–c). A slight concentration effect in clone 55 is

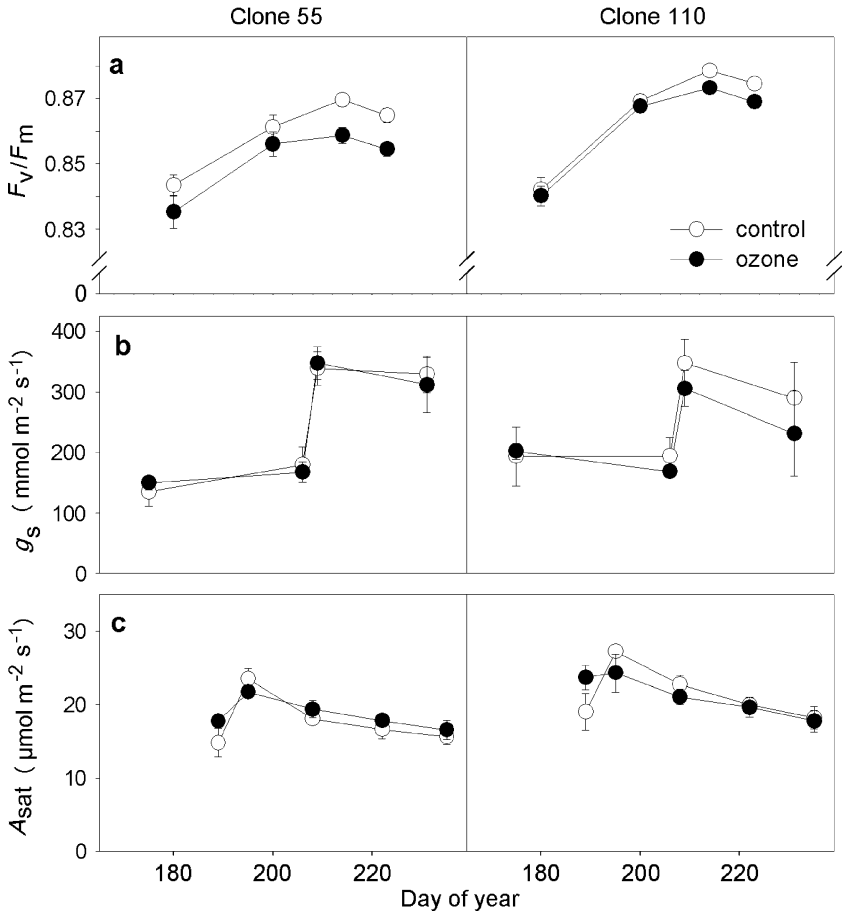


Fig. 4 Mean (**a**) maximum efficiency of PS II (F_v/F_m), (**b**) stomatal conductance (g_s), and (**c**) leaf-level net photosynthesis (A_{sat}) of two clones of soil-grown hybrid aspen saplings (N=4) during the third growing season in ambient ozone (*open circles*) and elevated ozone (*closed circles*). Error bars are SEs

seen in total flavonol glycosides (Fig. 5 e). Reduced accumulation of neochlorogenic acid, and considerably greater accumulation of chlorogenic acid in clone 55 compared with clone 110 was observed (Fig. 5 f–g).

Biomass and Mycorrhizal Root Status There were no significant differences in growth or dry mass in clones between the ozone treatments in the soil-grown trees (Table 4a). Instead, there were differences between the clones in all growth and biomass parameters (Table 4a). The total biomass of clone 110 was 65% greater than that of clone 55. The final height was 15% greater in clone 110, whereas the base diameter and diameter at 1.3 m were 34% and 40% greater, respectively, in clone 110 than in clone 55. The difference in the dry mass of fine roots (< 2 mm in diam) and coarse roots (> 2 mm) between ozone and control plots was not statistically significant (Table 5). The ergosterol content of the fine roots was significantly (41%) higher in elevated ozone plots than in control plots (Table 5).

We also reanalyzed the biomass data from the pot experiment of clones 55 and 110 (Table 4b). Even though clone 55 was clustered among the ozone-tolerant clones, and clone 110 among the ozone-sensitive clones (Häikiö et al. 2007), no effects of elevated ozone concentrations on the growth or biomass of either clone were observed, except for a statistically significant reduction in coarse root biomass in elevated ozone in clone 110 (Table 4b). Elevated ozone tended to reduce the stem dry mass more in clone 110 than in clone 55, and increased the final height of clone 55, whereas no effect of elevated ozone on the final height of clone 110 was observed (Table 4b). We found differences between the clones in the dry mass of stem and roots, and in the final base diameter, which were significantly greater in clone 110 than in clone 55 (Table 4b).

Principal Component Analysis PCA was used to study the relationships between the clones, different growth variables, and foliar phenolic composition (Fig. 6). The first PC explained almost 60% of the data, and was characterized by high scores in biomass attributes associated with clone 110 on the positive side, and the bulk of phenolic compounds associated with clone 55 on the negative side (Fig. 6). Growth was negatively correlated with catechins, condensed tannins, chlorogenic acid, and many quercetin derivatives (Fig. 6). No correlation between growth and A_{sat} or g_s was found (Fig. 6).

Table 3 The phenolic compounds of two clones of soil-grown hybrid aspen saplings in 2004

Peak		Clone 55						Clone 110						*P* values of main effects and interactions [a]		
		Ambient O_3			Elevated O_3			Ambient O_3			Elevated O_3					
		mg g^{-1}		SE	mg g^{-1}		SE	mg g^{-1}		SE	mg g^{-1}		SE	O_3	Clone	O_3 * clone
1	Salicin	4.70	±	0.79	3.47	±	0.38	3.74	±	0.43	3.55	±	0.63	0.371	0.282	0.211
4	Salicortin	26.45	±	5.15	35.98	±	1.39	19.36	±	6.20	29.17	±	6.30	0.202	**0.047**	0.960
8	Disalicortin	0.23	±	0.04	0.20	±	0.02	0.31	±	0.05	0.35	±	0.01	0.870	**0.003**	0.235
6	Tremuloidin	1.27	±	0.31	1.00	±	0.10	0.70	±	0.19	0.72	±	0.24	0.650	**0.040**	0.411
7	Tremulacin	29.96	±	9.50	37.51	±	3.17	14.45	±	5.76	25.56	±	8.75	0.369	**0.008**	0.634
	Total salicylates	*62.61*	±	*14.76*	*78.17*	±	*4.32*	*38.56*	±	*12.35*	*59.35*	±	*15.48*	*0.317*	***0.012***	*0.682*
3	(+)-catechin	7.61	±	0.95	10.74	±	1.29	2.38	±	0.73	3.77	±	0.64	0.111	**<0.001**	0.150
2	Gallocatechin	1.08	±	0.13	1.34	±	0.15	0.33	±	0.10	0.60	±	0.16	0.180	**<0.001**	0.966
–	Condensed tannins	56.45	±	15.30	67.79	±	11.70	16.39	±	8.32	26.21	±	7.35	0.505	**<0.001**	0.883
	Total tannins and catechins	*65.14*	±	*16.26*	*79.88*	±	*13.10*	*19.12*	±	*9.11*	*30.58*	±	*8.11*	*0.451*	***<0.001***	*0.771*
9	Chlorogenic acid	2.71	±	0.48	3.26	±	0.30	0.63	±	0.13	0.93	±	0.14	0.297	**<0.001**	0.569
8	Neochlorogenic acid	1.18	±	0.04	1.50	±	0.12	2.51	±	0.46	3.94	±	0.38	**0.037**	**0.001**	0.098
	Total phenolic acids	*3.88*	±	*0.52*	*4.76*	±	*0.40*	*3.14*	±	*0.59*	*4.87*	±	*0.43*	*0.092*	*0.242*	*0.132*
10	Quercetin-3-glycoside	0.11	±	0.01	0.11	±	0.01	0.08	±	0.01	0.08	±	0.00	0.681	**0.008**	0.967
14	Quercetin-3-arabinoglucoside	0.56	±	0.07	0.55	±	0.04	0.19	±	0.03	0.22	±	0.04	0.864	**<0.001**	0.530
15	Quercetin-3-arabinoglucuronide	0.47	±	0.04	0.48	±	0.05	0.35	±	0.02	0.35	±	0.01	0.875	**0.003**	0.895
16	Quercetin-3-galactoside	0.24	±	0.05	0.24	±	0.03	0.13	±	0.01	0.14	±	0.02	0.889	**0.007**	0.957
17	Quercetin-3-glucuronide + glucoside	3.06	±	0.32	2.99	±	0.28	2.18	±	0.17	2.24	±	0.13	0.991	**0.001**	0.669
19	Quercetin-3-arabinoside	0.11	±	0.02	0.10	±	0.00	0.09	±	0.01	0.10	±	0.02	0.940	0.564	0.658
11	Kaempferol digycoside	0.26	±	0.02	0.27	±	0.02	0.36	±	0.02	0.32	±	0.01	0.524	**0.001**	0.122
18	Kaempferol-3-arabinoglucoside	0.67	±	0.05	0.70	±	0.04	0.53	±	0.03	0.53	±	0.04	0.605	**0.002**	0.748
20	Kaempferol-3-glucuronide	2.03	±	0.28	2.05	±	0.13	2.33	±	0.20	2.43	±	0.12	0.811	**0.017**	0.717
12	Myricetin-3-galactoside	0.79	±	0.43	0.74	±	0.12	0.19	±	0.11	0.37	±	0.23	0.830	**0.043**	0.566
13	Myricetin-3-glucoside + glucuronide	0.29	±	0.01	0.51	±	0.17	0.90	±	0.03	0.80	±	0.06	0.551	**0.003**	0.131
21	Isorhamnetin-3-glucuronide	0.51	±	0.02	0.48	±	0.05	0.40	±	0.01	0.36	±	0.02	0.365	**0.001**	0.755
	Total flavonol glycosides	*9.08*	±	*1.23*	*9.21*	±	*0.66*	*7.72*	±	*0.64*	*7.93*	±	*0.53*	*0.876*	***0.027***	*0.931*
	Total phenolics	*140.7*	±	*32.6*	*172.0*	±	*16.1*	*68.5*	±	*22.4*	*102.7*	±	*22.5*	*0.284*	***0.003***	*0.733*

[a] The values are mean concentrations (mg g^{-1} DW) ± standard errors (SE) of four replicates. *P* values are from linear mixed model ANOVA

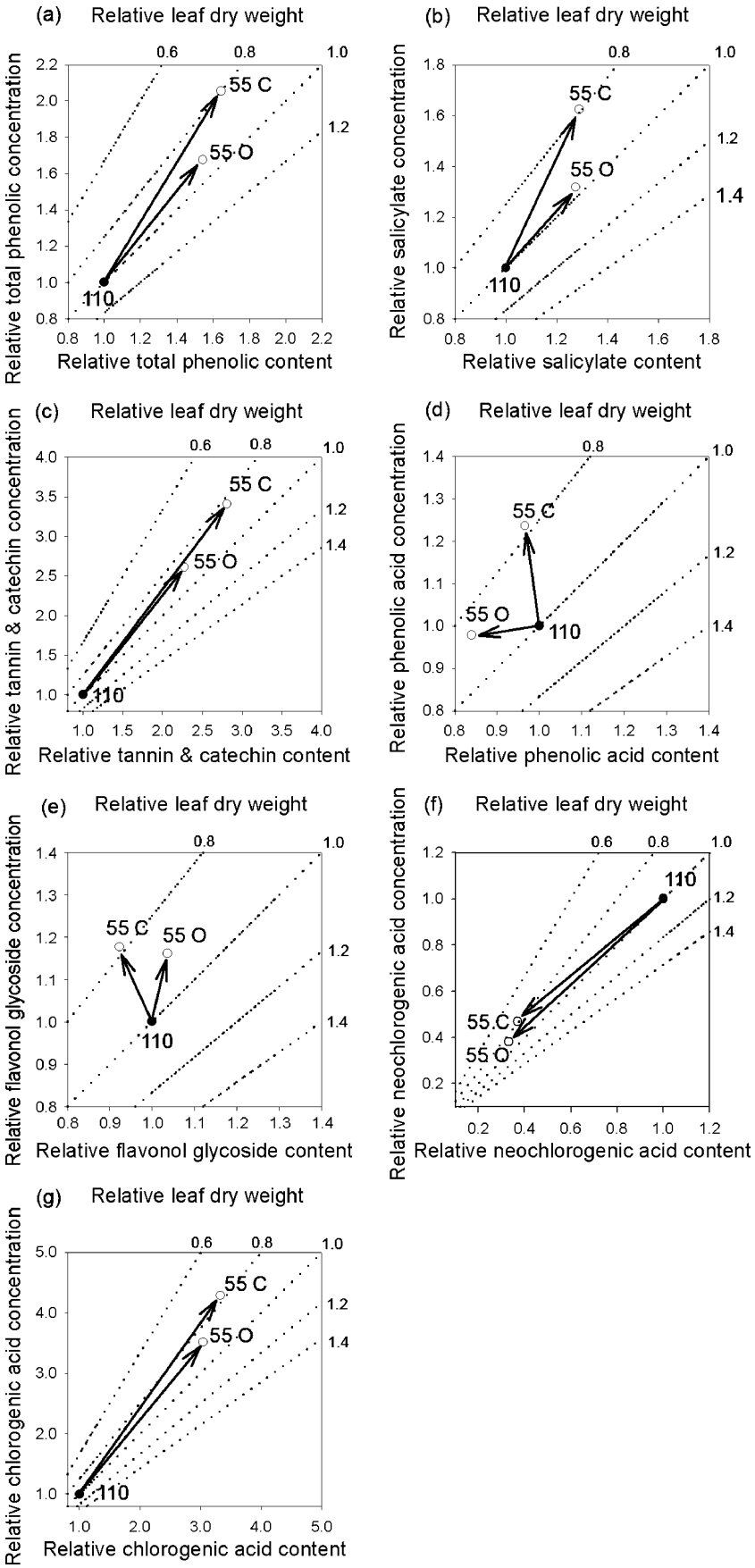


Fig. 5 Differences between two clones of soil-grown hybrid aspen saplings in the relative foliar concentration and content of (**a**) total phenolics, (**b**) total salicylates, (**c**) total tannins and catechins, (**d**) total phenolic acids, (**e**) total flavonol glycosides, (**f**) neochlorogenic acid, and (**g**) chlorogenic acid; and the relative leaf dry weight. The values for clone 110 were used as a reference point (1, 1, 1). Abbreviations: 55 C = clone 55 in ambient ozone; 55 O = clone 55 in elevated ozone. For interpretation of the vectors, see Fig. 3

Table 4 Dry mass, leaf area, height and base diameter of a) soil-grown saplings after three growing seasons, and b) potted saplings after two growing seasons of hybrid aspen clones 55 and 110 in ambient O_3 and elevated O_3

a) Soil-grown 2004				Main effects and interactions		
				P values		
	Clone	Ambient O_3	Elevated O_3	clone	O_3	clone x O_3
Dry mass of leaves (g)	55	44.8±6.8	38.4±2.5	**<0.001**	0.234	0.225
	110	126.7±20.1	97.9±6.1			
Dry mass of stem (g)	55	144.8±23.5	118.6±8.5	**<0.001**	0.280	0.357
	110	394.9±61.2	320.4±19.6			
Dry mass of coarse roots (g)	55	43.5±2.5	45.1±5.1	**<0.001**	0.249	0.189
	110	152.8±14.8	129.6±8.1			
Final height (cm)	55	291.2±19.3	278.1±9.4	**<0.001**	0.703	0.553
	110	336.7±23.6	332.0±7.9			
Final base diameter (mm)	55	18.9±1.2	18.0±0.4	**<0.001**	0.403	0.389
	110	29.0±2.4	26.6±0.5			
Diameter at 1.3 m (mm)	55	12.2±1.0	11.1±0.6	**<0.001**	0.358	0.624
	110	20.1±1.7	18.4±0.7			
b) Potted 2003				Main effects and interactions		
				P values		
	Clone	Ambient O_3	Elevated O_3	clone	O_3	clone x O_3
Dry mass of leaves (g)	55	19.0±1.2	20.0±1.1	0.948	0.677	0.207
	110	21.0±2.3	18.2±1.1			
Dry mass of stem (g)	55	68.2±1.7	67.2±4.3	**<0.001**	0.252	0.073
	110	96.8±5.4	81.2±5.8			
Dry mass of coarse roots (g)	55	35.7±1.9	29.6±2.3	**<0.001**	**0.042**	0.072
	110	58.6±3.8	41.4±4.6			
Final height (cm)	55	178.1±8.9	206.1±8.4	0.567	0.227	0.064
	110	194.8±7.5	197.1±6.3			
Final base diameter (mm)	55	14.5±0.2	13.9±0.4	**<0.001**	0.241	0.702
	110	15.9±0.4	15.1±0.5			

The values are means of four replicates ± SE. *P* values for the main effects and interactions of ozone and clone are from mixed model ANOVA

Discussion

Elevated Ozone Concentrations Did Not Affect the Competitive Ability of the Two Hybrid Aspen Clones Because our earlier studies with potted saplings of eight hybrid aspen clones showed significant ozone x clone interactions in most biomass attributes after two growing seasons (Häikiö et al. 2007), we expected to find differences in growth responses after three growing seasons between the more ozone-tolerant clone 55 and the more ozone-sensitive clone 110 growing in soil. However, no effects of elevated ozone in any of the growth parameters were found on either clone. Also, reanalysis of the potted saplings of clones 55 and 110 showed no significant differences in the growth responses to ozone, except for the dry mass of coarse roots showing 17% and 29% reductions in response to elevated ozone in clones 55 and 110, respectively. Significant differences in growth of both the potted and the soil-grown trees were found only between the two clones, not between the ozone treatments. This is in contrast with the observations of King et al. (2005) who found a 22% reduction in the standing biomass of trembling aspen after 7 years of ozone exposure. This might be due to the relatively low ozone enhancement and shorter time scale of our experiment compared with the studies of King et al. (2005). Clones 55 and 110 also were not among the most ozone tolerant or sensitive hybrid aspen clones used in our previous experiments (Oksanen et al. 2001; Häikiö et al. 2007). We also did not find changes in fitness in response to elevated ozone in the two hybrid aspen clones as shown by the lack of ozone x clone interaction in growth-associated parameters. Clone 110 was a better competitor than clone 55 in both ambient ozone and elevated ozone concentrations as shown by the greater biomass of clone 110 at the end of the experiment. Differences in growth responses to elevated ozone among clones of trembling

Table 5 The differences between ambient ozone and elevated ozone plots in the dry mass of fine roots (< 2 mm), coarse roots (> 2 mm) and ergosterol concentrations of fine roots

	Ambient O_3	Elevated O_3	*P* values
Fine roots (mg cm-3)	0.78±0.03	0.45±0.16	0.093
Coarse roots (mg cm-3)	1.22±0.27	0.97±0.48	0.666
Ergosterol (ng mg-1 DW)	606.0±39.1	856.4±81.4	**0.032**

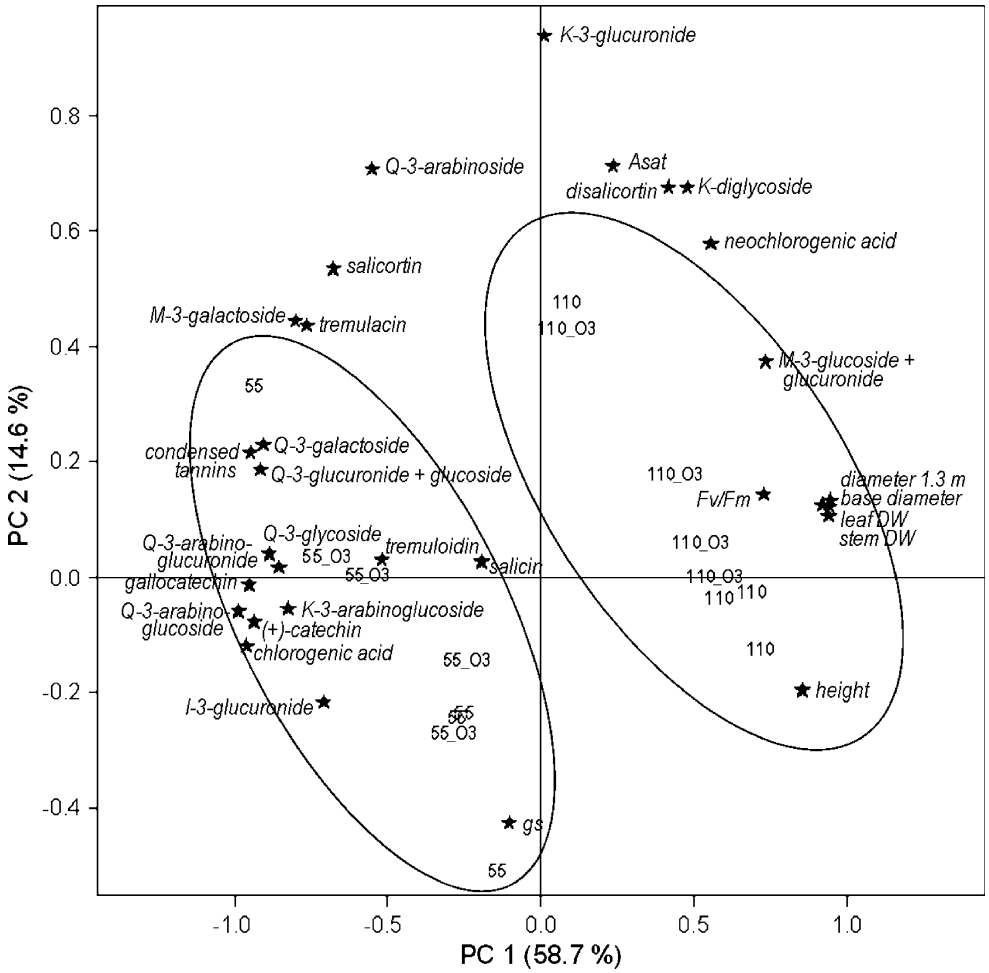


Fig. 6 The PCA biplot diagram showing the loading plot (*star symbols*) of phenolic compounds, biomass (leaf and stem dry weight, base diameter and diameter at 1.3 m, and final height), chlorophyll fluorescence (F_v/F_m), and gas exchange (A_{sat} and g_s), superimposed on the score plot of hybrid aspen clones indicated by clone numbers (each observation represents a mean value per clone per field). The variances explained by principal components 1 and 2 are shown in parentheses

aspen were reported by Karnosky et al. (2005), and after 7 years of ozone exposure, the extremely ozone-tolerant trembling aspen clone 8 L had a better competitive ability in elevated ozone than in the control treatment, whereas the poorest competitor was more strongly inhibited in elevated ozone than the other clones (Kubiske et al. 2007).

Elevated ozone concentrations usually reduce carbon allocation to roots (Grulke et al. 1998; Andersen 2003), which was also reported in our study with potted plants (Table 4b; Häikiö et al. 2007). Because of the difficulties in extracting coarse root systems of individual trees growing in soil, we determined the fine root biomass from each plot by collecting soil samples at random locations between the trees. Fine roots, which may comprise >75% of the root length in trees (Pregitzer et al. 2002), play the most important role in nutrient and carbon cycling in forests, even though they represent only a small fraction of total tree biomass. We did not find significant differences in fine root or coarse root dry mass between the ozone treatments. Consistent with our results, no changes in root biomass in response to elevated ozone concentrations were reported after two growing seasons in trembling aspen (King et al. 2001), or after three growing seasons in birch (Kasurinen et al. 2005). However, the response can change over time, and after eight growing seasons, increased fine-root biomass of trembling aspen in elevated ozone has been observed (Pregitzer et al. 2008). Increased numbers of both mycorrhizal and non-turgescent fine roots after 2 years of elevated ozone exposure in a mature beech stand were also reported by Grebenc and Kraigher (2007). Our root samples were collected on one sampling occasion at the end of August, and because fine root production is usually greater in spring and early summer than in autumn (Hendrick and Pregitzer 1996; Grulke et al. 1998), the responses may have been confounded by the temporal variation in fine root production and mortality. On the other hand, we did not observe a reduction in photosynthetic capacity of our clones in response to elevated ozone, and since the structural development of roots is dependent on current photosynthate (Andersen 2003), the lack of significant effects of ozone on root biomass is not surprising.

Ergosterol concentration of the fine roots was 41% higher in the elevated-ozone plots compared with control plots. Increases in the mycorrhizal status of the roots in response to elevated ozone has been reported in birch by Kasurinen et al. (2005) and in beech by Grebenc and

Kraigher (2007), who attributed the differences to changes in species composition and the relative abundance of ectomycorrhizal fungi. *Populus* species are colonized by both ectomycorrhizae (EM) and arbuscular mycorrhiza (AM; Smith and Read 1997; Neville et al. 2002), and ergosterol has been shown to be absent in AM fungi (Olsson et al. 2003). Therefore, ergosterol analysis can be used only for a coarse estimation of mycorrhizal biomass.

The nitrogen status of the soil-grown trees was good based on the high foliar nitrogen concentrations (> 30 mg g^{-1}) in both clones. Even though the trees were not systematically watered or fertilized during the three growing seasons, soil nutrient and water availability in the soil-grown trees were good compared with the potted plants, which probably suffered from both root restriction and drought, especially during the hot summer of 2003. When the final heights of the clones at the end of the second growing season were compared, no difference in height between potted and soil-grown trees was observed in clone 55 (P=0.550), whereas in clone 110, the soil-grown trees were 15% higher than the potted trees (P=0.002). This indicates that, when growing in soil, clone 110 was able to exploit the ample soil resources and increase its competitive ability. We found differences between the clones in the potted saplings in response to nitrogen amendment (Häikiö et al. 2007). There was a 26% increase in total dry mass in the high-N plants of clone 110, whereas no difference in total dry mass was found between the two nitrogen treatments in clone 55 (data not shown). This suggests that other resources than nitrogen restricted the growth of clone 55. Because *Populus* species are high isoprene emitters, volatile isoprene synthesis can lead to great losses of carbon fixed by photosynthesis (Monson and Fall 1989; Geron et al. 2001). In this experiment, however, no differences between the two clones in the concentrations of emitted isoprene were observed that could account for differences in carbon allocation for growth between the two clones (Blande et al. 2007).

High Concentrations of Leaf Phenolics Were Negatively Correlated with Growth Salicylates and condensed tannins comprise the majority of the phenolics in the leaves of *Populus* species, and have been studied in many experiments in context with herbivory, elevated ozone or elevated carbon dioxide (e.g., Holton et al. 2003; Agrell et al. 2005). We now have elucidated the whole phenolic profile, including the phenolic acids and flavonol glycosides, of hybrid aspen leaves in detail.

Previously, we reported high clonal variation in the foliar phenolic profile of eight hybrid aspen clones (Häikiö et al. 2007). The present study supports the finding that genotype and not ozone causes most of the variation in the secondary chemistry of the leaves (see also Kontunen-Soppela et al. 2007). Salicylates, condensed tannins, flavonol glycosides (derivatives of quercetin, kaempferol, and myricetin), and hydroxycinnamate derivatives may comprise up to one-third of the dry mass of aspen leaves (Tsai et al. 2006). Salicylates have been reported to be highly dependent on genotype, whereas the concentrations of condensed tannins vary across environmental conditions (Hemming and Lindroth 1999; Osier and Lindroth 2001, 2006). Higher concentrations of condensed tannins in elevated ozone were found in the foliage of loblolly pine (Jordan et al. 1991) but in a meta-analysis, no effects of elevated ozone alone on the concentrations of condensed tannins were reported (Valkama et al. 2006). In this study, we detected no effect of ozone on either salicylates or condensed tannins, which is in contrast with our previous study with potted trees, where higher concentrations of condensed tannins and their precursor (+)-catechin, as well as chlorogenic acid, were found in ozone-fumigated hybrid aspen saplings (Häikiö et al. 2007). Instead, the only phenolic significantly increased by elevated ozone in the soil-grown plants was neochlorogenic acid, which was more abundant in clone 110 than in clone 55. The caffeoyl moiety of chlorogenic acid or neochlorogenic acid may act as a precursor to lignins, and ozone-induced increases in lignins, which may act as barriers or have an antioxidant effect on reactive oxygen species, have been reported in *Populus* leaves and stem wood (Cabane et al. 2004; Kaakinen et al. 2004). The chlorogenic acids are also powerful antioxidants (Grace et al. 1998), and increased levels of chlorogenic acid under ozone stress have been observed in birch (Saleem et al. 2001; Peltonen et al. 2005).

The clonal differences in the foliar phenolic profiles can be seen easily in the PCA biplot (Fig. 6), where most phenolics are associated with clone 55. Clone 110 had 30% lower concentrations of salicylates, 66% lower concentrations of catechins and condensed tannins, and 15% lower concentrations of flavonol glycosides than clone 55. GVA showed that the higher concentrations of phenolics in clone 55 were the result of increased synthesis and not the result of concentration due to smaller leaf dry weight. As indicated by PCA, high concentrations of phenolics, especially catechins and condensed tannins, were negatively correlated with growth. A negative correlation between growth and condensed tannins was also found by Donaldson et al. (2006) and Häikiö et al. (2007). The biosynthetic costs of condensed tannins or salicylates are not very high when presented as grams of glucose required per gram of compound (Gershenzon 1994), but considering the high concentrations of these compounds in the leaves, the net cost of production of phenolics of clone 55 was twice as high as that of clone 110. Even though elevated ozone concentrations did not affect the competitive ability of the clones, the slower growth of clone 55 compared with clone 110 may have led to an increase in resource limitation and a decrease

in competitive ability, which could have resulted further in greater allocation to secondary compounds instead of growth. In our studies, phenolics comprised 19% of the dry weight of the leaves in the potted trees, compared with 12% in the soil-grown trees. This may be explained by nutrient limitation (P, K) or drought stress which may have shifted carbon allocation towards phenolics in the potted plants (Donaldson et al. 2006). Clone 55 was also infected by a fungal disease (tentatively identified as *Marssonina* blight) from the start of the experiment, which likely affected the secondary chemistry of the leaves. In *Melampsora*-infected hybrid poplar, the condensed tannin pathway was induced (Miranda et al. 2007), and in *Salix*, enhanced synthesis of catechin, salicortin, and chlorogenic acid in response to *Melampsora* infection have been reported (Hakulinen 1998; Hakulinen et al. 1999).

To summarize, elevated ozone concentrations did not affect the gas exchange or the final dry mass of the two hybrid aspen clones studied, indicating that the clones did not differ in their ozone sensivitity. This was confirmed by renanalyzing clonal data from a previous experiment with potted plants of the same clones. There were no changes in the ozone sensitivity affecting the competitive ability of either clone after 3 years of moderately elevated ozone exposure. Instead, high carbon allocation to phenolics (mostly condensed tannins and salicylates) resulted in poor growth and significant differences in the final biomass between the two clones. Higher resource availability resulted in markedly lower foliar phenolic concentrations in the soil-grown trees compared with the potted trees of the same clones.

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Effects of Phenylcarboxylic Acids on Mitosis, Endoreduplication and Expression of Cell Cycle-Related Genes in Roots of Cucumber (*Cucumis sativus* L.)

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Abstract Several benzoic and cinnamic acid derivatives were identified from cucumber root exudates. The effects of these phenylcarboxylic acids on root growth and cell cycle progression were examined in germinated seeds of cucumber. All 12 phenylcarboxylic acids (0.25 mM) tested significantly inhibited cucumber radicle growth, and cinnamic acid exerted a dose-dependent inhibitory effect. At 6 h after exposure to the acids, transcript levels of the cell cycle-related genes, including two cyclin-dependent kinases (CDKs) and four cyclins were reduced. Among them, transcript of *CycB*, a marker gene for mitosis showed a remarkable reduction. The temporal analysis showed that expression of mitotic genes (*CDKB*, *CycA*, and *CycB*) were reduced throughout the experiment, while the reduction of the other genes (*CDKA*, *CycD3;1*, and *CycD3;2*) were observed only at earlier time points. At 48 h after treatment with benzoic and cinnamic acids, an enhancement of endoreduplication was observed. Further time course analysis indicated that endoreduplication started as early as 6 h after exposure to cinnamic acid. These results provide evidence that exposure to benzoic and cinnamic acids can induce rapid and dramatic down-regulation of cell cycle-related genes, thus leading to root growth inhibition. Meanwhile, the block of mitosis caused by phenylcarboxylic acids also induced an increased level of endoreduplication.

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Keywords Allelopathy · Phenylcarboxylic acid · Cell cycle · Cyclin · CDKs · Endoreduplication

Introduction

Plants synthesize an array of chemicals that are involved in a variety of plan-plant, plant-microbe, and plant-herbivore interactions (Inderjit and Duke 2003; Weir et al. 2004). The allelochemicals that are responsible for plant-plant allelopathy are delivered into the rhizosphere mainly through leaching, volatilization, decomposition, and root exudation (Rice 1984; Singh et al. 1999). Allelopathy is usually interspecific (Weidenhamer et al. 1989; Callaway and Aschehoug 2000), but also may occur within the same species, which is called autotoxicity (Singh et al. 1999, Yu et al. 2000). The problem of autotoxicity is common in croplands, and is one of the major reasons for growth reduction in monocropping (Singh et al. 1999; Yu et al. 2000). Although autotoxicity has long been recognized, little is known about its exact action mode.

Investigation of the chemical composition of allelopathic plants reveals that a great number of secondary metabolites serve as allelochemicals. Among them, benzoic and cinnamic acid derivatives are frequently identified from soils or root exudates (Yu and Matsui 1994; Blum 1996; Inderjit and Duke 2003). Root growth inhibition by benzoic and cinnamic acids has been widely observed (Chon et al. 2002; Iqbal et al. 2004; Hiradate et al. 2005; Rudrappa et al. 2007; Batish et al. 2008). Furthermore, exogenous addition of allelochemicals can influence physiological and biochemical reactions, such as photosynthesis, respiration, water and nutrient uptake, and generation of reactive oxygen species (Hejl et al. 1993, Hejl and Koster 2004; Gonzalez et al. 1997; Yu and Matsui 1997; Ding et al.

2007), as well as induce changes in gene expression (Golisz et al. 2008). All such physiological, biochemical, and transcriptional changes are implicated directly or indirectly in plant growth inhibition.

Plant growth ultimately is driven by the process of cell division coupled with the subsequent expansion and differentiation of the resulting cells (Beemster et al. 2003; Jakoby and Schnittger 2004). Cell division plays a role both in the developmental processes that create plant architecture and in the modulation of plant growth rate in response to the environment (Cockcroft et al. 2000; Beemster et al. 2002). The normal cell cycle mode is characterized by a round of DNA replication (S phase) followed by mitosis and cytokinesis (M phase), and separated by two gap phases (G_1 and G_2) (Inzé 2005). Cyclin-dependent kinases (CDKs) and their cyclin partners regulate the G_1/S- and G_2/M-phase transitions as well as progression through and exit from the cell cycle (Menges et al. 2002; Beemster et al. 2005; Inzé and De Veylder 2006). Studies have shown that regulation of cell cycle-related gene expression in different phases is an important mechanism for control of progression through the cell cycle (Menges et al. 2002; Beemster et al. 2005). At present, little is known about the relationship between allelopathy and cell division. Cell cycle progression has been proposed as a target (Burgos et al. 2004; Nishida et al. 2005; Sánchez-Moreiras et al. 2008). However, no data are available concerning the influence of allelochemicals such as phenylcarboxylic acids on the regulation of cell cycle progression by investigation of cell cycle-related gene expression.

Poor plant growth in monocropping of cucumber is supposedly related to autointoxication partly arising from root exudates (Yu et al. 2000). Phenylcarboxylic acids including benzoic and cinnamic acids have been identified from root exudates of cucumber plants, and they had detrimental effects on ion uptake and enhanced the incidence of *Fusarium* wilt by triggering oxidative stress in cucumber (Yu and Matsui 1994, 1997; Ye et al. 2004, 2006; Ding et al. 2007). This study was performed to investigate the influences of benzoic and cinnamic acids on cucumber root growth and cell cycle progression in radicle tips.

Methods and Materials

Plant Material Cucumber (*Cucumis sativus* L. cv. Jinyan No.4) seeds were germinated at 25°C in darkness. Fifty seeds were imbibed in petri dishes (diameter=15 cm) on two layers of Whatman filter paper saturated with 15 ml distilled water. When the radicle had begun to emerge, germinating seeds were transferred to another petri dish with different solutions of derivatives of benzoic and cinnamic acid (benzoic, vanillic, *p*-hydroxybenzoic, *o*-hydroxybenzoic, 3,4-dihydroxybenzoic, gallic, cinnamic, 3-phenylpropionic, caffeic, *p*-coumaric, ferulic, and sinapic acids) at 0.25 mM for another 48 h. Distilled water served as the control. To study concentration effect of cinnamic acid, germinating seeds were transferred to petri dishes containing cinnamic acid at 0.05, 0.1, 0.25, and 0.5 mM for another 48 h. Radicle length was determined at 48 h after exposure to different phenylcarboxylic acids, and inhibition rates of radicle length were calculated. Each experiment was carried out with three independent replicates.

Nuclei DNA Content Analysis We analyzed the relative nuclei DNA content of cucumber radicle tips (0–2 mm) at 48 h after treatments with different kinds of derivatives of benzoic and cinnamic acids (0.25 mM). Afterwards, changes in nuclei DNA content with different concentrations of cinnamic acid (0.05, 0.1, 0.25, and 0.5 mM) were investigated in the radicle tips of cucumber seeds at 48 h after treatment. Additionally, temporal changes in nuclei DNA content of radicle tips were determined at 6, 24, and 48 h after treated with cinnamic acid (0.25 mM). Samples were chopped with a razor blade in 1 ml of ice-cold buffer (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS, pH=7, and 1% Triton X-100) (Galbraith et al. 1983), filtered over a 45-μm nylon mesh, and stained with 4,6-diamidino-2-phenylindole. Stained nuclei were analyzed by a FACSCalibur flow cytometer (Becton, Dickinson and Company, USA). Calibration of C-values was made with nuclei from young leaves of cucumber.

Data were analyzed with CellQuest software to determine the ploidy distribution. Mean C-value (MCV) was calculated as the sum of the number of nuclei of each ploidy level multiplied by its endoreduplication cycle and divided by the total number of nuclei (Barow and Meister 2003), where C-value represents the DNA content of the monoploid chromosome set of an organism (Greilhuber et al. 2005; Greilhuber 2008).

RNA Extraction Total RNA was extracted from 0–2 mm radicle tips of cucumber with different treatments with TRIZOL reagent (Sangon, China) according to the manufacturer's instructions at various time after treatments. After extraction, total RNA was dissolved in diethyl pyrocarbonate-treated water.

Transcript Level Estimation with qRT-PCR Quantitative real-time PCR (qRT-PCR) assays were performed to determine the relative transcript level of each cDNA. Gene-specific primers for qRT-PCR were designed based on EST sequences for six cell cycle-related genes, *CDKA* (cyclin-dependent kinase A), *CDKB* (cyclin-dependent kinase B), *CycA* (A-type cyclin), *CycB* (B-type cyclin),

CycD3;1 (D-type cyclin), and *CycD3;2* (D-type cyclin). The specific sets of primers used for the amplification of each cDNA are summarized in Table 1. The first-strand cDNA used as template for qRT-PCR was synthesized by using a RevertAid^{TM} first strand cDNA Synthesis Kit (Fermentas) from 2 µg of total RNA purified by using a RNeasy Mini Kit (Qiagen).

qRT-PCR was performed with an iCycler iQ Multicolor Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA). Each reaction (20 µl total volume) consisted of 10 µl iQ SYBR Green Supermix, 1 µl of diluted cDNA, and 0.1 µM of forward and reserve primers. PCR cycling conditions were as follows: 95°C for 3 min, 40 cycles of 95°C for 10 s, and 58°C for 45 s. Fluorescence data were collected during the 58°C step. The cucumber *actin* gene was used as an internal control. Relative gene expression was calculated as described by Livak and Schmittgen (2001).

Statistical Analysis Seeds were arranged in three randomized blocks with three replicates per treatment. All results were subject to analysis of variance (Statistica 6.0, Microsoft Corp., 1995), and the means were compared by the Tukey' HSD test at a significance level of $P \leq 0.05$.

Results

Effects of Benzoic and Cinnamic Acids on Radicle Elongation of Cucumber The effects of benzoic acids (benzoic, vanillic, *p*-hydroxybenzoic, *o*-hydroxybenzoic, 3,4-dihydroxybenzoic, and gallic acids) and cinnamic acids (cinnamic, 3-phenylpropionic, caffeic, *p*-coumaric, ferulic, and sinapic acids) at 0.25 mM on cucumber radicle elongation were examined at 48 h after treatments (Table 2). Exposure to these acids all resulted in a reduction in radicle length. The inhibition rates ranged from 20.69 to 59.43%, as compared to the control treated with distilled water. Among the 12 acids assayed, cinnamic acid had the greatest inhibitory effect while benzoic acid had the lowest. Comparatively, benzoic acids showed less inhibitory effects (20.7–36.0%) than cinnamic acids (34.8–59.4%). Additionally, the concentration effect of cinnamic acid, an important autotoxin of cucumber, on cucumber radicle growth was investigated. Cinnamic acid at 0.05 mM had only slight inhibitory effects on radicle elongation while cinnamic acid at 0.1 mM significantly inhibited radicle length (Fig. 1). Exposure to cinnamic acid at 0.25 mM and 0.5 mM resulted in a more marked reduction of radicle length by 52.4% and 69.3%, respectively.

Effects of Benzoic and Cinnamic Acids on Relative Nuclei DNA Content in Cucumber Radicle Tips To examine the effects of benzoic and cinnamic acids on mitosis and endoreduplication in cucumber radicle tips, nuclei DNA content was measured by flow cytometric analysis in cucumber radicle sections 2 mm apart in the radicle tip, the zone with most active cell division in the radicle. The first peak of nuclei observed in the histogram for cucumber was defined as 2C, and the following peaks were 4C, 8C, and so on according to Gilissen et al. (1993). Nuclei with 2, 4, and 8C were all found in control radicle tips, and the MCV was relatively constant (3.83–3.99) (Tables 3, 4, and 5). As shown in Table 3, all tested benzoic and cinnamic acids significantly decreased the proportion of 2C but increased the proportion of 8C. Among them, radicles exposed to sinapic, caffeic, and cinnamic acids showed higher 8C values than those exposed to other acids. However, these acids had little effect on the proportion of 4C. The MCV increased from 3.96 for the control to

Table 1 Primers used for quantitative real-time PCR assays

Gene	Encoding protein	Accession No.	Primer pairs
CycA	A-type cyclin	EW968279	F: GATTTGTTCGTGCTGCTCAA
			R: TAATTCGGCAAGGAAGTTGG
CycB	B-type cyclin	EW968280	F: GAGAACGAGAGCAGACCTCA
			R: CAATCTTTGTCGCAAGGAAA
CycD3;1	D-type cyclin	EW968283	F: CATGTGGAGGAGAAAGCAGA
			R: TTGATGCTGACACAATCGTC
CycD3;2	D-type cyclin	EW968284	F: CTATCAACCCTCACAAACGC
			R: TGTTGCTCTAAATGCCCAAG
CDKA	cyclin-dependent kinase A	EW968281	F: TCTTCGTGGCATTGCATATT
			R: TCTTGCCAGTCCAAAGTCAG
CDKB	cyclin-dependent kinase B	EW968282	F: CAATCCCTCTATGTCGTTCG
			R: GCTTGAGATCACGGTGAAGA
actin		DQ641117	F: AAAGATGACGCAGATAAT
			R:GAGAGATGGCTGGAATAG

Table 2 Inhibition of radicle elongation of cucumber plants exposed to 0.25 mM phenylcarboxylic acids for 48 h

Phenylcarboxylic Acids	Inhibition Rate of Radicle Length (%)
Control	0.0 g
Benzoic Acid	20.7 f
Vanillic Acid	35.2 d
p-Hydroxybenzoic Acid	30.4 e
o-Hydroxybenzoic Acid	36.0 d
3,4-Dihydroxybenzoic Acid	33.2 de
Gallic Acid	33.0 de
Cinnamic Acid	59.4 a
3-Phenylpropionic Acid	48.6 b
Caffeic Acid	43.4 c
p-Coumaric Acid	34.8 d
Ferulic Acid	35.5 d
Sinapic Acid	48.2 b

Numbers with different letters refer to significant differences among the treatments ($P \leq 0.05$). The concentration of each phenylcarboxylic acid was 0.25 mM. Each treatment was carried out using three independent biological replicates.

4.43 and 4.66 after exposure to benzoic and cinnamic acids, respectively. Accordingly, exposure to all these acids resulted in an increased level of endoreduplication in radicle tips.

Table 4 shows that cinnamic acid at 0.05 mM had no significant influence on the MCV but significantly decreased the proportion of 2C. When the concentration of cinnamic acid reached ≥0.1 mM, the proportion of 2C decreased and the proportion of 8C increased. The MCV increased by 14.7%, 17.8%, and 23.5% in the 0.10, 0.25, and 0.5 mM cinnamic acid treatments, respectively, as compared to the control. A time course study showed that exposure to cinnamic acid at 0.25 mM resulted in a decrease in 2C proportion and an increase in 8C proportion from 6 h after treatment, leading to a significant increase in MCV. Compared with the control, the MCV index increased by 6.0, 11.5, and 10.2% at 6, 24, and 48 h after treatment with 0.25 mM cinnamic acid, respectively (Table 5).

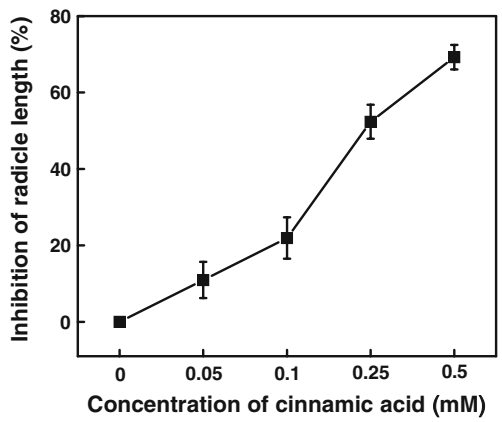

Fig. 1 Effects of different concentrations (0, 0.05, 0.1, 0.25, and 0.5 mM) of cinnamic acid on inhibition of cucumber radicle elongation (%) at 48 h after treatment

Effects of Benzoic and Cinnamic Acids on Cell Cycle-related Genes Expression To gain insight into the mechanism by which these acids regulate the cell cycle, the expression of six cell cycle-related genes, *CDKA*, *CDKB*, *CycA*, *CycB*, *CycD3;1*, and *CycD3;2* were examined in cucumber radicle tips (2 mm) after treatments. Transcript levels of all examined genes were reduced in radicles after exposure to 0.25 mM benzoic, vanillic, *p*-hydroxybenzoic, *o*-hydroxybenzoic, cinnamic, 3-phenylpropionic, and *p*-coumaric acids. Exposure to 3,4-dihydroxybenzoic and gallic acids reduced the transcript level of *CycB* but had negligible effects on transcript levels of other genes (Fig. 2). Caffeic acid had little effect on transcript levels of *CDKA*, *CycA*, and *CycD3;1* but greatly reduced transcript levels of *CDKB*, *CycB*, and *CycD3;2*. Similarly, ferulic and sinapic acids reduced the transcript levels of *CDKA*, *CDKB*, *CycB*, *CycD3;1*, and *CycD3;2* but had negligible effects on transcript levels of *CycA* (Fig. 2).

We further analyzed the changes in transcript levels of cell cycle-related genes in cucumber radicles in response to different concentrations of cinnamic acid at 6 h after treatment, as well as at various time after treatment with 0.25 mM cinnamic acid. As shown in Fig. 3, transcript levels of all six cell cycle-related genes decreased with the increase in the concentration of cinnamic acid. Transcript levels of *CDKA*, *CDKB*, *CycA*, *CycB*, *CycD3;1*, and *CycD3;2* decreased to 61.2, 57.7, 62.2, 20.5, 61.5, and 52.71% of the control, respectively, in radicles after 6 h-exposure to 0.25 mM cinnamic acid (Fig. 3). The inhibition in the expression of six cell cycle-related genes was detectable earlier (at 3 h) after exposure to 0.25 mM cinnamic acid (Fig. 4). The expression of *CDKA* gene was repressed at 3 h after exposure to cinnamic acid, and a more dramatic decrease was observed at 6 h after treatment. Transcript levels of *CDKB*, *CycA*, and *CycB* were down-regulated throughout the treatment time. *CycD3;1* and

Table 3 Effects of benzoic and cinnamic acids on relative nuclei DNA content and mean c-value in cucumber radicle tips at 48 h after treatment

Phenylcarboxylic Acids	2C (%)	4C (%)	8C (%)	Mean C-Value
Control	35.3a	48.1	16.6b	4.0b
Benzoic Acid	25.6b	50.5	23.9a	4.4a
Vanillic Acid	25.6b	50.4	24.0a	4.4a
p-Hydroxybenzoic Acid	26.9b	49.1	24.1a	4.4a
o-Hydroxybenzoic Acid	24.8b	50.4	24.8a	4.5a
3,4-Dihydroxybenzoic Acid	23.8b	49.5	26.7a	4.6a
Gallic Acid	26.7b	48.2	25.2a	4.5a
Cinnamic Acid	25.2b	47.9	27.0a	4.6a
3-Phenylpropionic Acid	27.2b	49.2	23.6a	4.4a
Caffeic Acid	24.1b	48.1	27.8a	4.6a
p-Coumaric Acid	26.2b	48.6	25.2a	4.5a
Ferulic Acid	27.5b	48.1	24.4a	4.4a
Sinapic Acid	23.4b	48.3	28.3a	4.7a

Numbers with different letters in the same column refer to significant differences among the treatments ($P \leq 0.05$). The proportions of 2C, 4C, and 8C nuclei were measured on 10 000 nuclei. The concentration of each phenylcarboxylic acid was 0.25 mM. Each treatment was carried out using three independent biological replicates.

CycD3;2 genes were down-regulated at 3 and 6 h, and then, their transcript levels increased gradually (Fig. 4).

Discussion

In the present study, we used benzoic and cinnamic acid derivatives identified from cucumber root exudates (Yu and Matsui 1994; Asao et al. 1999), to investigate their impact on cucumber root growth and cell cycle regulation. Germinated seeds were used since radicles or the young roots usually are the first organs to obtain contact with autotoxic agents. The results showed that all phenylcarboxylic acids caused strong inhibition in cucumber radicle elongation (Table 2), which is consistent with previous studies (Yu and Matsui 1994, 1997; Yu et al. 2003; Dos Santos et al. 2008; Batish et al. 2008; Tharayil et al. 2008). Cinnamic acid, an important autotoxin of cucumber, showed a higher inhibitory effect than other compounds, as has been observed in other plants (Yu and Matsui 1997; Hiradate et al. 2005). It is worth noting that cinnamic acid was applied at reasonable concentrations (0~0.5 mM) in this study since the concentration of autotoxins was 0.1 mM in soils after cucumber cultivation and may reach much higher levels in soils enriched in plant residues (An et al. 2001a, 2001b; Yu et al. unpublished data). The presence of a variety of autotoxins exuded from cucumber roots likely would exhibit synergism (Yu and Matsui 1994; An et al. 2001b). All tested compounds caused significant inhibition in radicle length to different degrees (Table 2), which was possibly due to chemical characteristics such as water solubility (Yu and Matsui 1997; Tharayil et al. 2008).

The elaboration of plant form and function depends on the ability of a cell to divide and differentiate (Inzé 2005). Benzoic and cinnamic acids differently induced down-regulations of two CDKs (*CDKA* and *CDKB*) and four cyclin (*CycA*, *CycB*, *CycD3;1*, and *CycD3;2*) genes at the transcript level (Figs. 2, 3, and 4). Among them, expression of *CycB*, a marker gene for mitosis and specific to the G_2/M phase of the cell cycle (Hemerly et al. 1992; Ferreira et al. 1994), was the most reduced, suggesting that the transition of G_2 to M was blocked by the phenylcarboxylic acids; thus, the ability of cells to enter cell division was impaired (Cools and De Veylder 2009). This is consistent with a report that benzoxazolin-2(*3H*)-one selectively retards cell cycles at the G_2/M checkpoint in lettuce seedling root meristems (Sánchez-Moreiras et al. 2008).

Table 4 Effects of different concentrations of cinnamic acid on relative nuclei DNA content and mean c-value in cucumber radicle tips at 48 h after treatment

Concentrations (mM)	2C (%)	4C (%)	8C (%)	Mean C-Value
0	33.7a	52.4a	13.9c	3.9c
0.05	29.8b	52.4a	16.5c	4.0c
0.1	28.2b	50.0a	23.6b	4.4b
0.25	25.2b	47.9a	27.0a	4.6b
0.5	25.8c	45.7b	30.5a	4.8a

Numbers with different letters in the same column refer to significant differences among the treatments ($P \leq 0.05$). The proportions of 2C, 4C, and 8C nuclei were measured on 10 000 nuclei. Each treatment was carried out using three independent biological replicates.

Table 5 Effects of cinnamic acid on relative nuclei DNA content and mean c-value in cucumber radicle tips at different time after treatment

Hours (hr)	Treatments	2C (%)	4C (%)	8C (%)	Mean C-Value
6	Control	39.2a	45.3	15.4b	3.8b
	Cinnamic Acid	33.6b	48.2	18.3a	4.1a
24	Control	36.4a	46.0	17.6b	4.0b
	Cinnamic Acid	26.1b	46.9	27.0a	4.6a
48	Control	35.8a	48.5	15.8b	3.9b
	Cinnamic Acid	28.3b	49.6	22.2a	4.3a

Numbers with different letters in the same column refer to significant differences between the treatments ($P \leq 0.05$). The proportions of 2C, 4C, and 8C nuclei were measured on 10 000 nuclei. The concentration of cinnamic acid is 0.25 mM. Each treatment was carried out using three independent biological replicates.

It also has been demonstrated that salt stress and fungal elicitors also induce the repression of the B-type cyclin gene expression (West et al. 2004; Suzuki et al. 2006). Similar to *CycB* gene, expression of *CycA* gene, an A-type cyclin involved in the control of S-to-M phase, was reduced by the phenylcarboxylic acids. Possibly, cinnamic and other acids induce generation of reactive oxygen species that suppress the transcript level of *CycA*, since the expression of *CycA* gene is sensitive to oxidative stress (Reichheld et al. 1999; Ding et al. 2007). *CDKB* gene is specific to the G_2 and M phases (Magyar et al. 1997; Porceddu et al. 2001) while the *CDKA* gene plays a pivotal role in both the G_1-to-S and G_2-to-M transition points, and its activity is rate limiting for the plant cell cycle (Hemerly et al. 1995; Inzé 2005). Similar to other studies under salt stress, water stress, and cold night conditions (Setter and Flannigan 2001; West et al. 2004; Rymen et al. 2007), the expression of *CDKA* and *CDKB* genes was down-regulated by the tested phenylcarboxylic acids, especially *CDKB*, which showed a constant down-regulation pattern during the treatment time. This also suggests that mitosis was more seriously inhibited.

D-type cyclins are thought to regulate the G_1-to-S transition (Menges et al. 2006). The reduced transcript levels of *CycD3;1* and *CycD3;2* in cucumber radicles after exposure to phenylcarboxylic acids suggested that they

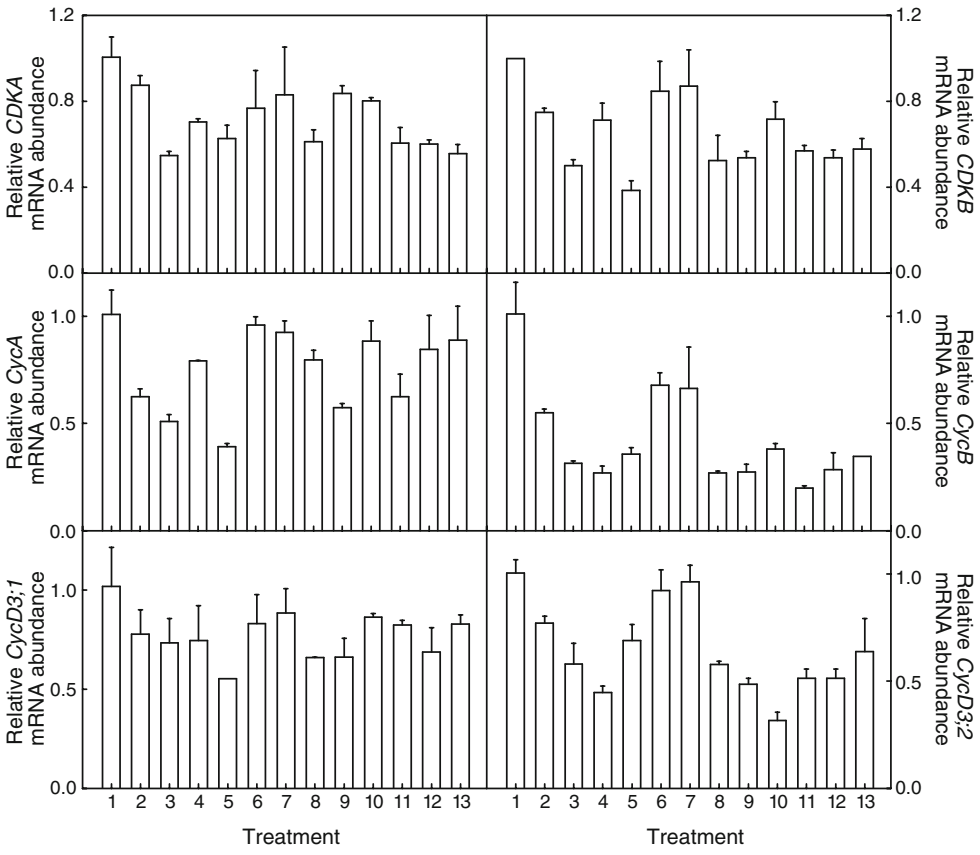

Fig. 2 Expression of cell cycle-related genes in cucumber radicle tips at 6 h after exposure to 12 phenylcarboxylic acids. The numbers of the 1–13 displaying on the horizontal axis represent different treatments with distilled water, benzoic acid, vanillic acid, *p*-hydroxybenzoic acid, *o*-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, gallic acid, cinnamic acid, 3-phenylpropionic acid, caffeic acid, *p*-coumaric acid, ferulic acid, and sinapic acid, respectively. The concentration of each phenylcarboxylic acid was 0.25 mM. Expression levels by qRT-PCR are expressed as a ratio of the control, which is set as 1

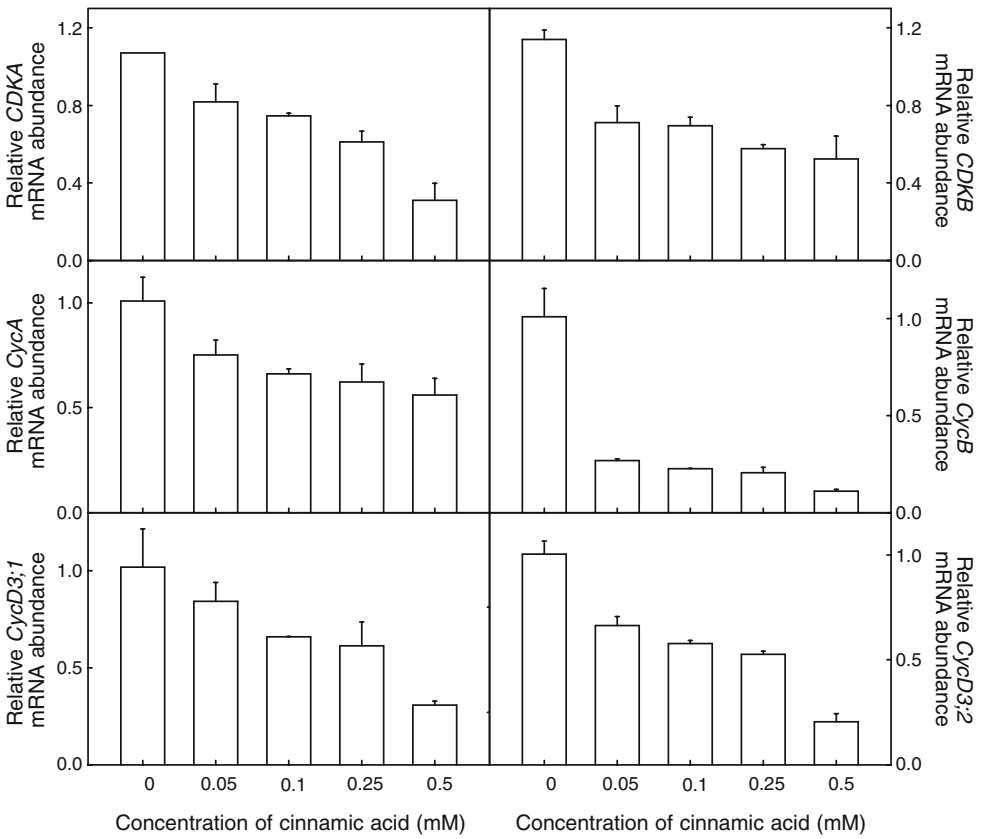

Fig. 3 Expression of cell cycle-related genes in cucumber radicle tips at 6 h after exposure to different concentrations of cinnamic acid. The concentrations of cinnamic acid used were 0.05, 0.1, 0.25, and 0.5 mM, respectively. The control was treated with distilled water. Expression levels by qRT-PCR are expressed as a ratio of the control, which is set as 1

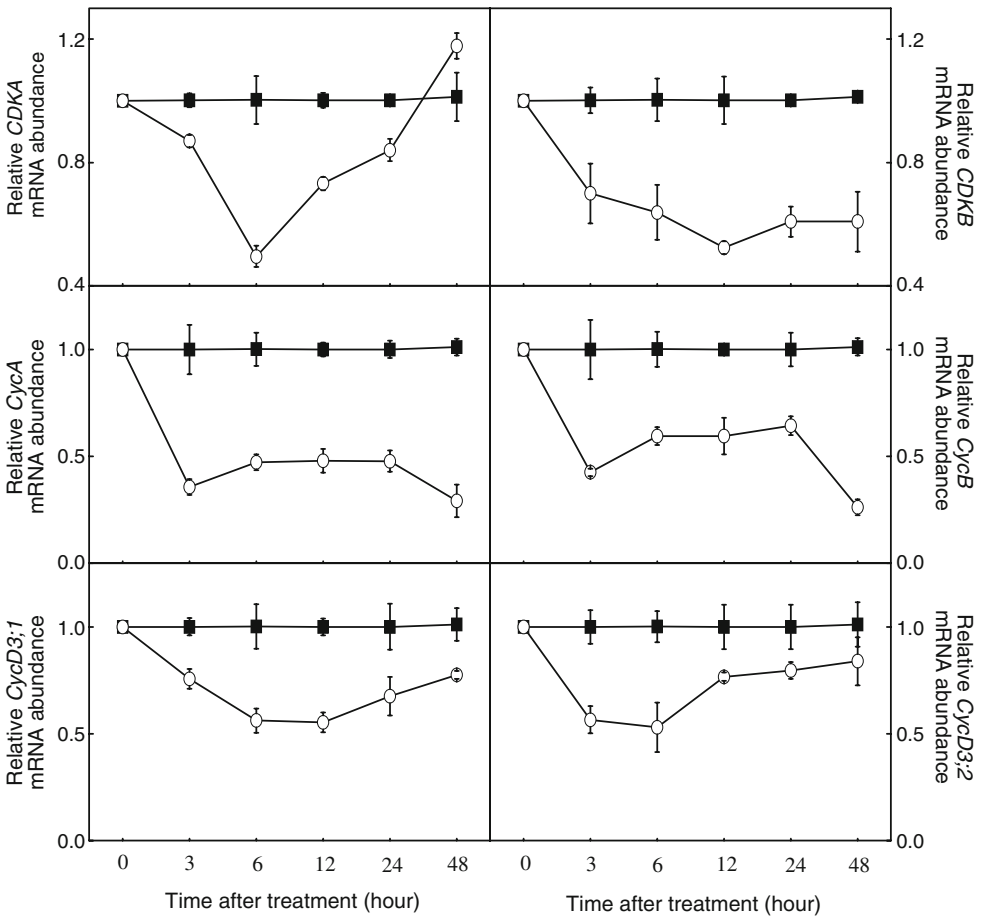

Fig. 4 Expression of cell cycle-related genes in cucumber radicle tips at different hours after exposure to cinnamic acid. The concentration of cinnamic acid was 0.25 mM. Expression levels by qRT-PCR are expressed as a ratio of the control, which is set as 1. The control (filled rectangular), 0.25 mM cinnamic acid (open circle)

altered the G_1-to-S transition process. However, it is interesting to note that both the expression of *CDKA* and *CycD3* genes increased from 12 h after treated with 0.25 mM cinnamic acid, which was different from the expression patterns of *CDKB*, *CycA*, and *CycB* genes in our study (Fig. 4). It is likely that *CDKA* and *CycD3* genes also play roles in the process of endoreduplication since they are active in the G_1/S phase (see below).

The existence of 2, 4, and 8C cells in cucumber radicles suggested the occurrence of endoreduplication, as observed in a previous study (Gilissen et al. 1993). Endoreduplication is another mode of the cell cycle. The mitotic cell cycle comprises the duplication and subsequent distribution of chromosomes between two daughter cells, whereas endoreduplication involves repetitive chromosomal DNA replication and marks the exit from the cell division program, leading to an increase in the ploidy level (Joubès and Chevalier 2000; Cools and De Veylder 2009). In this study, we found that all the tested phenylcarboxylic acids induced higher levels of endoreduplication compared with the control. The enhancement of endoreduplication that occurred after phenylcarboxylic acids treatments was possibly a protective and adaptive mechanism to environmental stress as has been observed in cadmium stress (Fusconi et al. 2006; Repetto et al. 2007).

Previous studies have shown that senescence can induce a higher level of endoreduplication, and both benzoic and cinnamic acids can trigger the generation of reactive oxygen species and ultimately cause loss of root viability (Galbraith et al. 1991; Ding et al. 2007). It is possible that the induction of significant amounts of reactive oxygen species by cinnamic acid could consequently result in the process of early senescence and cause a higher level of endoreduplication. Down-regulation of mitotic genes induced by benzoic and cinnamic acids then may directly result in a different distribution of the nuclei class of ploidy in the differentiated roots. *CDKB* and *CycB* genes are actively involved in the cell mitosis phase, whereas reduced expression of these genes is accompanied by the onset of enhanced endoreduplication (Schnittger et al. 2002; Boudolf et al. 2004; Cools and De Veylder 2009). In our study, *CDKB* and *CycB* were significantly and constantly repressed at the transcript levels when exposed to phenylcarboxylic acids (Figs. 2, 3, and 4), indicating a significant inhibition of the mitotic phase. Since the mitotic phase was blocked, the initiation of the endoreduplication phase was enhanced (Tables 3, 4, and 5).

In summary, this study demonstrated that benzoic and cinnamic acid derivatives caused rapid and dramatic down-regulation of cell cycle-related genes in cucumber radicles, leading to radicle growth inhibition. Meanwhile, they induced an increased degree of endoreduplication due to the blocking of mitosis. This is possibly a protective mechanism against stress.

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Two Regulatory Mechanisms of Monoterpenoid Pheromone Production in *Ips* spp. Of Bark Beetles

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Abstract Bark beetles use aggregation pheromones to coordinate host colonization and mating. These monoterpenoid chemical signals are produced *de novo* in midgut cells via the mevalonate pathway, and pheromone production is induced when an adult beetle feeds on phloem of a host tree. In *Ips pini*, juvenile hormone (JH) III influences key regulatory enzymes along the mevalonate pathway that leads to pheromone production. In fact, topically applied JH III is sufficient to stimulate pheromone production in unfed males. In this study, we explore the influence of feeding and JH III treatment on pheromone production in male *Ips confusus,* the pinyon Ips. We also characterize the influence of feeding and JH III treatment on transcript levels and activity of three key enzymes involved in pheromone biosynthesis: 3-hydroxy-3-methylglutaryl-CoA (HMG) synthase (HMGS), HMG-CoA reductase (HMGR) and geranyl diphosphate synthase (GPPS). We also extend the current understanding of the regulation of pheromone biosynthesis in *I. pini,* by measuring the influence of feeding and JHIII treatment on enzymatic activity of HMGS and GPPS. Feeding on host phloem alone strongly induces pheromone production in male *I. confusus*, while JH III treatment has no effect. However, feeding and JH III both significantly up-regulate mRNA levels of key mevalonate pathway genes. Feeding up-regulates these genes to a maximum at 32 h, whereas with JH III-treatment, they are up-regulated at 4, 8, and 16 h, but return near to non-treatment levels at 32 h. Feeding, but not JH III treatment, also increases the activity of all three enzymes in *I. confusus,* while both feeding or treatment with JH III increase HMGS and GPPS activity in *I. pini.* Our data suggest that pheromone production in *Ips* is not uniformly controlled by JH III and feeding may stimulate the release of some other regulatory factor, perhaps a brain hormone, required for pheromone production.

Keywords *Ips confusus* · *Ips pini* · Pheromones · Juvenile hormone · Mevalonate pathway

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Introduction

In *Ips* species, production of aggregation pheromones is induced when a pioneering adult beetle feeds on host phloem while attacking a tree. If a sufficient number of beetles respond to these chemical signals, host defenses can be overcome and the tree colonized. For example, male *I. confusus* produce a pheromone of monoterpenoid alcohols ipsenol, ipsdienol, and *cis*-verbenol (Birch et al. 1977). In *I. pini,* ipsenol and ipsdienol are produced *de novo* in midgut tissues via the mevalonate pathway (Seybold et al. 1995b; Ivarsson et al. 1998; Hall et al. 2002a). It is clear that in *I. pini* and other *Ips* species juvenile hormone (JH) III influences key regulatory enzymes along the mevalonate pathway that leads to pheromone production (see Fig. 1; Byers and Birgersson 1990; Ivarsson et al. 1993; Seybold et

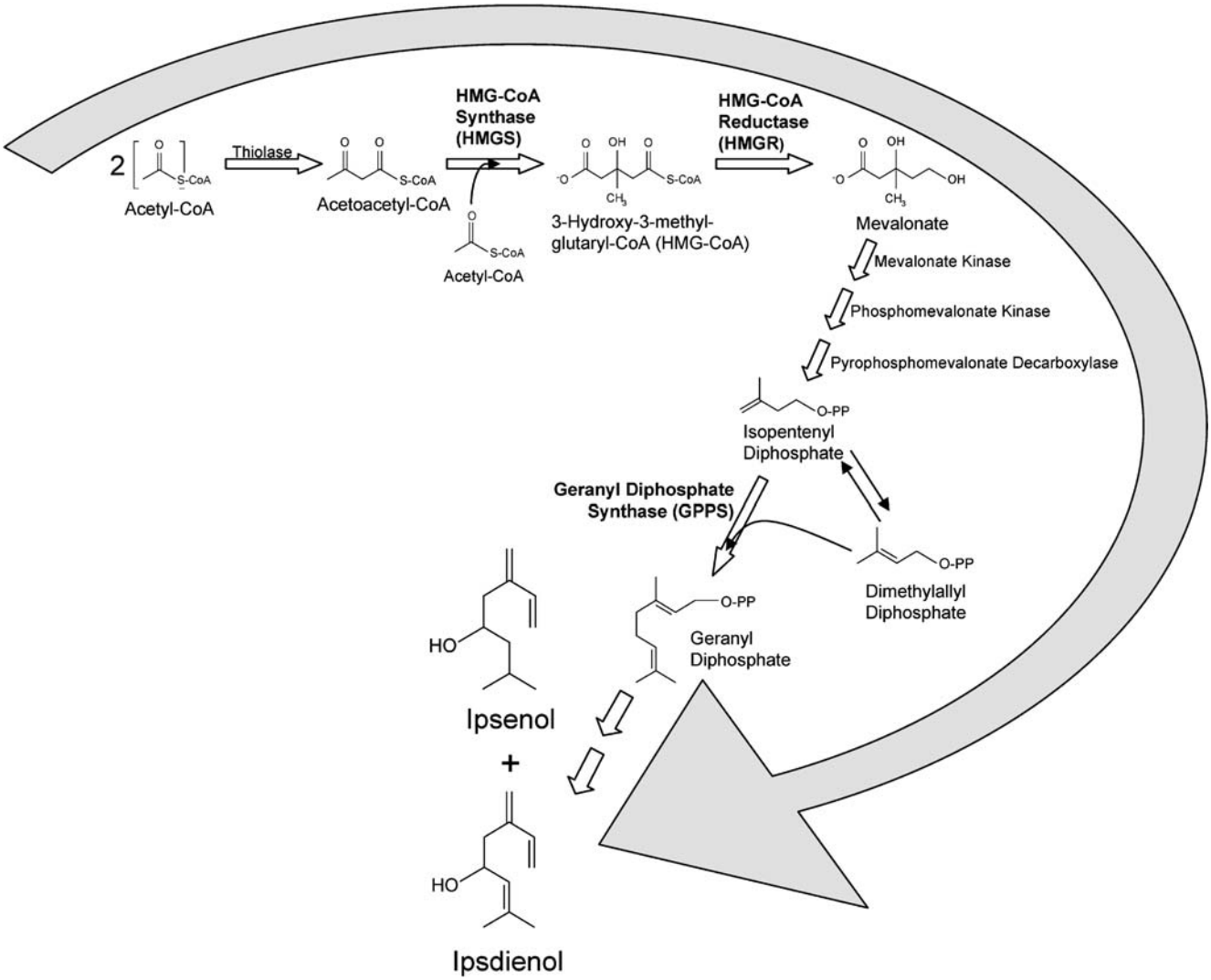

Fig. 1 Mevalonate pathway genes and associated enzymes involved in biosynthesis and regulation of pheromone production in *Ips* spp

al. 1995a; Seybold et al. 1995b; Tillman et al. 1998). In male *I. paraconfusus* and *I. pini,* JH III regulates mRNA levels of 3-hydroxy-3-methylglutaryl-CoA (HMG) reductase (HMGR) (Tittiger et al. 1999), the first committed and a highly regulated step in the mevalonate pathway. Moreover, feeding and JH III treatment strongly upregulate levels of HMGR transcript in the anterior midgut tissues of *I. pini* (Keeling et al. 2004). From these studies, it appears that in *I. pini* feeding on host phloem stimulates JH III synthesis by the *corpus allatum*, which, in turn, increases the expression level and enzyme activity of HMGR in the mevalonate pathway resulting in high levels of pheromone production (Tillman et al. 1998). In addition, JH III also upregulates transcription of other mevalonate pathway genes, including HMG-CoA synthase (HMGS) and geranyl diphosphate synthase (GPPS), in male *I. pini* (Gilg et al. 2005; Bearfield et al. 2006; Keeling et al. 2006).

Although JH III strongly regulates HMGR activity in *I. pini*, it has no influence on enzymatic activity in other *Ips* species. In assays measuring the conversion of ^{14}C-HMG-CoA to ^{14}C-mevalonolactone by fed and JH III treated males in several species, JH III treatment caused large increases in HMGR activity in *I. pini* but had no effect in *I. paraconfusus* (Tillman et al. 2004). However, feeding strongly increased the enzymatic activity of HMGR in both species; suggesting that members of the *grandicollis* subgeneric group (*e.g., I. paraconfusus*) are quite different from *I. pini* with regard to the regulation of pheromone production by juvenile hormone. Interestingly, *Ips confusus* is closely related to *I. paraconfusus* and falls into the same *grandicollis* group based on multiple gene analysis (Cognato and Vogler 2001) and mitochondrial cytochrome oxidase I DNA sequences alone (Cognato and Sperling 2000).

In this study, we use biochemical and molecular approaches to test the hypothesis that JH III does not exclusively regulate pheromone production in *Ips confusus,* a member of the *grandicollis* subgeneric group. We also evaluate for the first time the effect of JH III treatment and feeding on the transcriptional regulation of key mevalonate pathway genes in male *I. confusus*.

Materials and Methods

Insects Immature *I. confusus* were obtained from infested *Pinus monophylla* bolts collected from the Bureau of Land Management (BLM) land east of Carson City, Nevada, USA. We also collected bolts of *Pinus jeffreyi* infested with immature *I. pini* from the University of Nevada Whittell Forest, NV and from within the Lake Tahoe Basin Management Unit, South Lake Tahoe, CA. The insects were reared to adults in a greenhouse according to Browne (1972) and emerged adults were collected daily. Adult beetles were separated by sex according to Lanier and Cameron (1969) and stored for up to two weeks at 4°C in moist paper towels until used in experiments.

Influence of JH and Feeding on the Production of Key Pheromone Components We assessed the influence of JH III on the production of key pheromone components, ipsenol and isdienol, by collecting headspace volatiles of adult beetles on an absorbent. Individual male beetles (N= 30) were topically treated with either 10 μg of racemic JH III (Sigma-Aldrich, St. Louis, MO) dissolved in 0.5 μl of acetone or acetone alone (control) applied to the abdominal venter. Each group of beetles was then placed in a modified glass septum-inlet adaptor (29/32 joint, Sigma-Aldrich) and an absorbent filter containing 100 mg of 50/80 mesh Super Q® (Alltech Associates, Deerfield, IL) was attached to one end of the chamber with a 3 cm section of Tygon® tubing. Humidified and charcoal-purified air was pulled through the apparatus at a rate of ~500 ml/min. The beetles were aerated in the dark for 20 hr and then the Super Q® filters were eluted with 1 ml of pentane: ether (1:1) spiked with *n*-octanol (20 μg), as an internal standard. The resulting extracts were analyzed by gas chromatography (GC) using a Hewlett-Packard 5890 Series II GC equipped with an HP-INNOWax (J&W Scientific) capillary column (30 m × 0.25 mm × 0.25 μm film thickness) in splitless mode with a constant column flow of 1.5 ml He/min. The oven temperature was ramped from 60 to 150°C at 5°C/min, then 150 to 240°C at 10°C/min with a 5 min hold at 240°C. The identity of ipsenol and ipsdienol present in the samples was confirmed by comparing their retention times to those of authentic standards.

To determine the influence of feeding on the production of ipsenol and ipsdienol by male *I. confusus,* we treated 30 male beetles with 0.5 μl of acetone each and then placed them into individual 4 mm holes drilled through the bark of a small bolt of pinyon pine (~10 cm diameter and 15 cm long). The infested bolt was then placed in an inert oven cooking bag supported by a cylindrical wire cage (25 cm height, 20 cm diameter) and the beetles were allowed to feed. Humidified and charcoal-purified air was drawn out of the bag (~1 l/min) and volatile compounds were collected for 20 hr on an absorbent filter made of 100 mg of 50/80 mesh Super Q®. As a control, we also collected volatiles from an uninfested bolt of pinyon pine into which 30 holes had been drilled. The filters were then eluted and the resulting extract analyzed by GC as described above. The amount of ipsenol and ipsdienol produced by beetles in each treatment was compared by one-way ANOVA, followed by Fisher's least significant difference (LSD) test (STATSOFT 2005; Sokal and Rohlf 1995). We sampled volatiles three times for each treatment.

Gene Expression Analysis by Real-time PCR Male *I. confusus* were either treated with JH III (10 μg in 0.5 μl acetone), fed on *P. monophylla* phloem, or treated with 0.5 μl of acetone alone (control), or left untreated. All insects were kept at room temperature throughout the experiment. After 4, 8, 16, and 32 hr, the anterior midguts were excised from the beetles in water, purged of their contents, frozen immediately in liquid nitrogen and stored at -80°C. Each treatment was replicated six times for each time point with five midguts were used per replicate per time point.

Total RNA was prepared from the midgut tissue using the RNeasy Mini plant kit (Qiagen). A portion of the RNA was reverse-transcribed into first strand cDNA template using Superscript III RNase H- reverse transcriptase and random hexamer primers (Invitrogen). *Ips confusus cytoplasmic actin* was chosen as the endogenous control gene because it is unresponsive to JH III treatment or feeding in *I. pini* midguts (Keeling et al. 2004, 2006). After cloning *cytoplasmic actin* from *I. confusus*, semi-quantitative RT-PCR was used to confirm its lack of response to JH III or feeding. Moreover, further studies using quantitative real time (qRT-PCR) also confirmed that *I. confusus cytoplasmic actin* is a suitable normalizing gene (Sandstrom et al. 2008). Fragments of *I. confusus* HMGR, HMGS, and GPPS were isolated using degenerate primers based on previously reported sequences, and then sequenced to design specific real-time primers. All gene-specific primers were chosen only after first being identified with the Primer Express software program (Applied Biosystems, version 2.0) and then analyzed using Vector NTI Advance software (Invitrogen, version 9.1) to decrease the possibility of primer dimer formation. PCR amplifications were performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) following the manufacturer's protocol. Each

reaction was prepared in a separate well containing cDNA template, 300 nM of both forward and reverse primers and SYBR Green PCR Master Mix (Applied Biosystems). Relative gene expression was determined using the ΔΔCT method (Livak and Schmittgen 2001).

Influence of JH III Treatment and Feeding on Enzyme Activity of HMGS, HMGR, and GPPS We assessed the enzymatic activity of HMGS, HMGR and GPPS in groups of male *I. confusus* treated with 0.5 µl of acetone and allowed to feed in a bolt of pinyon pine for 4, 8 and 16 hr. Beetles were placed in holes predrilled into the phloem and strips of aluminum screen were secured over the holes to prevent the insects from leaving the log. Males were also treated with JH III (10 µg JH III in 0.5 µl of acetone) and incubated in the dark at room temperature for 4, 8 and 16 hr. The same number of beetles treated with 0.5 µl of acetone and incubated in the dark served as controls for each time point. We also measured the enzymatic activity of HMGS and GPPS in male *I. pini* treated as described above. The enzymatic activity of HMGR in response to feeding and JH III treatment has recently been reported for *I. pini* (Tillman et al. 2004) and was not further studied here.

HMG-CoA Synthase Activity Midguts of ~ 30 individual beetles for each treatment were dissected, stored in liquid nitrogen and homogenized with a mortar and pestle in 20 ml of homogenization buffer (0.25 M sucrose, 0.1 mM EDTA, 2.0 mM Hepes, and 100 mM of PMSF, pH 7.2). Microsomes were separated by differential centrifugation and enzyme activity assayed as described by Clinkenbeard et al. (1975) and revised by Scharnagl et al. (1995). In this assay, 100 µg of microsomal protein was combined with 120 µm acetoacetyl-CoA in 200 µl of assay buffer (0.1 M Tris-HCl (pH 8.0), 0.1 mM EDTA and 20 mM $MgCl_2$). The reaction mixture was then incubated for 2 min in a 30°C water bath and 400 µM total acetyl-CoA (~55,500 dpm/reaction) was added. The reaction then remained in the water bath and after 9 min (linear range) was stopped by pipetting a 50 µl aliquot of the mixture into scintillation vials containing 200 µl of 6 N HCl. The tubes were then placed in a 90°C sand bath for 2 hr. Under these conditions, CoA thioesters are hydrolyzed and non-reacted [^{14}C] acetate is evaporated, and only [^{14}C] HMG acid, from enzymically formed HMG-CoA, remains. The amount of labeled HMG acid formed during the reaction by HMG-CoA synthase was then determined using a TRI-CARB 2900 TR liquid scintillation analyzer. Samples were run in triplicate and, as an appropriate control, we also measured the amount of radiolabeled HMG acid formed in reactions that contained no enzyme protein.

HMG-CoA Reductase Activity HMG-CoA reductase activity was assayed using a protocol modified from Casals et al. (1996) and Tillman et al. (2004). In this assay, midguts were dissected from ~ 90 adults for each treatment, placed in liquid nitrogen, and homogenized in 500 µl of assay buffer (100 mM sucrose, 40 mM K_2PHO_4, 30 mM EDTA, 50 mM KCl, 10 mM DTT, pH 7.4). Microsomes were then isolated by serial centrifugation (HMGR protein is located in the endoplasmic reticulum). The tissue homogenate was first centrifuged at 7740×*g* for 5 min at 4°C to remove large debris, and the resultant supernatant was further centrifuged at 12,100×*g* for 20 min to remove the mitochondria. To pellet the microsomal fraction, the supernatant from the 12,100 x *g* spin was ultracentrifuged at 165,000×*g* for 69 min at ~ −5ºC. The resulting pellet was then resuspended in 20 µl of assay buffer (50 mM K_2PHO_4, 5 mM EDTA, 200 mM KCl, 5 mM DTT, pH 7.4) and total protein concentration was determined by the Bradford method (1976) using bovine serum albumin (1 mg/ml) as an external standard.

3-Hydroxy-3-methylglutaryl-CoA reductase activity was determined by measuring the conversion of [3-^{14}] HMG-CoA to [3-^{14}] mevalonolactone. Specifically, the reaction mix contained 175 µM total HMG-CoA (1:1 labeled: unlabeled; ~44,000 dpm), 10 mM NADPH, ~ 100 µg of microsomal protein in 200 µl of assay buffer (N=3 assay samples from one preparation of microsomes). The mixture was incubated for 10 min in a 37ºC water bath with shaking and then the reaction was terminated with the addition of 20 µl of 5 M HCl. The samples were then incubated for another 30 min in the 37ºC water bath to catalyze the cyclization of linear mevolonate to mevalonolactone (MLL). Following the incubation, the reaction mixture was extracted three times with 5 ml aliquots of diethylether which were combined over Na_2SO_4. The combined ether extracts were then concentrated to ~ 200 µl and the [^{14}C] mevalonolactone was separated from the unreacted [^{14}C] HMG-CoA by silica paper chromatography using a solvent development system of 1:1 hexane:acetone. A MLL standard was also run along with the radiolabeled reaction products. The migration (R_f value) of the radiolabeled compounds along the silica plate were determined using a BioScan Imaging Scanner System 200-IBM (Bioscan, Inc., Washington, D.C.). The position of the [^{14}C]mevalonolactone on the silica plate was determined by comparing its R_f value to that of the MLL standard. The portion of silica containing the labeled MLL was then scraped from the plate and the radioactivity was quantified using a Packard TRI-CARB 2900 TR (Perkin Elmer, Germany) liquid scintillation analyzer (LSA).

GPPS Activity Prenyltransferase activity was measured as per Gilg et al. (2005) with modifications. A standard acid-lability assay was used to measure the incorporation of [1-^{14}C] isopentenyl diphosphate (IPP) into polyprenyl

products. Specifically, ~ 100 µg of soluble beetle tissue extract (~30 beetles per treatment) was combined with allylic substrate (50 µm DMAPP) and 10 µM [1-^{14}C] total IPP (55 µCi/µmol) in 200 µl of assay buffer (20 mM Hepes (pH 7.2), 2.5 mM $MgCl_2$, 5 mM KF, 1 mM DTT, 10% glycerol, and protease inhibitor). The reaction mixture was then incubated for 1 h at 32ºC. The resulting acid was hydrolyzed by adding10 µl of 5 N HCl and reincubating the mixture for 30 min at 32ºC. After the incubation period, the reaction products were extracted with 300 µl ddH_2O and 1 ml hexane and the radioactivity of 500 µl of the organic/hexane phase radioactivity measured using a Packard TRI-CARB 2900 TR (Perkin Elmer, Germany) liquid scintillation analyzer (LSA).

We also used reverse phase-high performance liquid chromatography (HPLC) to confirm that isoprenyl diphosphate was the product of the reaction. The reaction was carried out as described above, but terminated by boiling the samples for 5 min and then spinning them at 16,000×*g* for 5 min. Clear homogenates (injection volume 200 µl: 100 µl of reaction sample mixed with 75 µl of buffer and 25 µl of authentic mixed standards (DMAPP, GPP, FPP, GGPP; 1:1:0.5:0.5) were directly loaded onto a Discovery 250×4.6 C_{18}, 5 µm, silica column (Supelco) using a HP 1050 series HPLC. Phosphorylated short-chain isoprenyls were cleanly separated using a solvent system of 25 mM NH_4HCO_3 pH 7.0: acetonitrile as described by Zhang and Poulter (1993). Samples were eluted at a flow rate 1 ml/min by using a linear gradient program: 100% 25 mM NH_4HCO_3 for 5 min and 0–100% acetonitrile for 30 min. Eluent was monitored by UV (214 nm). One milliliter fractions were collected by using an Amersham Pharmacia LKB-FRAC-100 Fraction Collector (Amersham Pharmacia Biotech), and fractions corresponding to IPP/DMAPP,GPP, FPP, and GGPP peaks (retention times 4, 16, 20, and 25 min, respectively) were assayed for radioactivity by using a TRI-CARB 2900 TR LSA.

Statistical Analysis of Enzyme Activity The activity of HMGS, HMGR, and GPPS in response to feeding and JH III treatment was expressed as a fold increase in enzymatic activity compared to acetone-treated controls (see Schiefelbein et al. 2008). Data are reported as means±S.E. and enzymatic activity over time by treatment was compared using a nested analysis of variance (nested ANOVA, Dunnett's method; STATSOFT 2005).

Results

Influence of Feeding and JH on the Production of Key Pheromone Components GC analysis of volatiles collected from fed and JH III-treated male *I. confusus* revealed that ipsenol and ipsdienol are produced only in response to feeding (one-way ANOVA, $F_{4,\ 10}$=45.6, P<0.0001). In response to feeding for 20 h, males released 563.4±31.1 ng of ipsenol (mean±SE) and 194.3±65.2 ng of ipsdienol; these data suggest that JH III alone is not sufficient to stimulate pheromone production in male *I. confusus.*

Expression Analysis Partial cDNA clones were obtained for *I. confusus* HMGR (IcHMGR; accession no. FJ536869), HMGS (IcHMGS; accession no. FJ536868), GPPS (IcGPPS; accession no. FJ536870), and *cytoplasmic actin* (IcActin; accession no. FJ536867) using *I. pini* gene-specific primers and degenerate primers in conjunction with homology-based PCR techniques. The effect of a 10 µg dose of JH III on the mRNA levels of the three mevalonate pathway genes in the anterior midgut of male beetles was determined at multiple time points using quantitative real-time PCR. In male *I. confusus*, feeding and JH III treatment were both sufficient to raise transcript levels for the three genes examined (Fig. 2). For male beetles, each gene demonstrated slightly different transcript values at each time point, but the expression patterns over time were very similar. In general, the mRNA levels following JH III-treatment showed a modest increase and peaked at either 8 or 16 hr and gradually decreased thereafter, reaching a level at or below the 4 hr time point at 32 hr post-treatment (Fig. 2). In comparison, transcript levels following feeding demonstrated a similar pattern as JH III treatment at the first two time points, but the induction was not as pronounced. Whereas the induction of the mevalonate pathway genes begins to decline at the latter time points (16 and 32 hr) with JH III treatment, feeding causes a marked increase in mRNA levels, reaching their highest values at 32 hr post treatment.

HMG-CoA Synthase Activity In both male *I. confusus* and *I. pini* there was a significant increase in enzyme activity over time by treatment (ANOVA, *I. confusus,* $F_{6,\ 18}$=168.6, P< 0.001; *I. pini*, $F_{6,\ 18}$=22.9, P<0.001; Fig. 3). In male *I. pini,* both JH III and feeding caused significant increases in HMGS activity over the acetone-control at the 16 hr time point (2.32±0.89 and 4.23±0.32 fold increases, respectively; ANOVA, Dunnett's method, P<0.001). However, only feeding alone caused a significant increase in enzymatic activity over the acetone-treated control in male *I. confusus* (4.85±0.25 fold increase; ANOVA, Dunnett's method, P<0.001).

HMG-CoA Reductase Activity There was a significant increase in enzymatic activity of HMGR with time by treatment (ANOVA, $F_{6,\ 17}$=3.65, P<0.05; Fig. 4), and feeding caused a significant increase in enzyme activity over the control at the 8 and 16 hr time points (ANOVA,

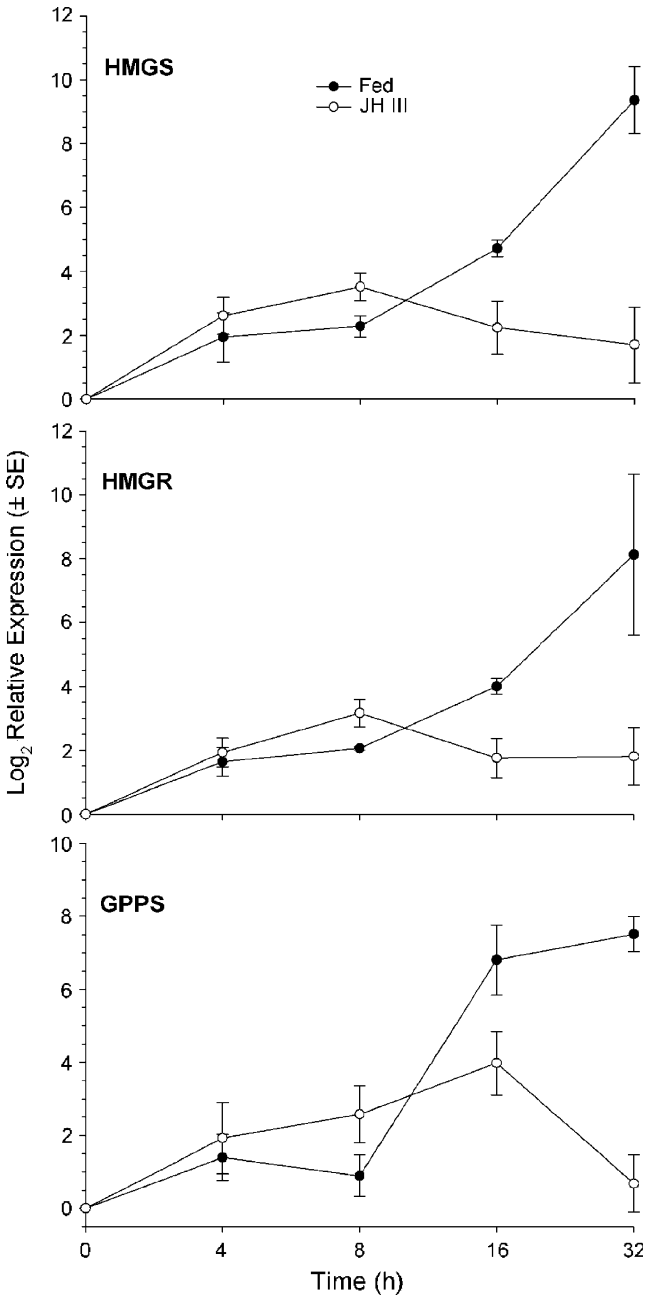

Fig. 2 Effect of JH-III treatment and feeding on the relative expression of *HMGS* (top), *HMGR* (middle), and *GPPS* (bottom) in midguts of male *I. confusus* as measured by qRT-PCR. Expression (Log_2) is relative to either acetone-treated males for JH-III treatment or unfed controls for fed males

Dunnett's method, $P < 0.001$ and $P < 0.05$, respectively). In fact, HMGR activity was increased by 1.32±0.29 fold over the control at 8 h and 0.86±0.41 fold at 16 hr. Treatment with JH III, however, caused no significant increase in activity over the acetone treated controls at all time points (Fig. 4).

GPPS Activity GPPS activity increased with time by treatment for both *I. confusus* and *I. pini* (ANOVA, *I. confusus,* $F_{6,\ 18}=8.85$, $P<0.001$; *I. pini*, $F_{6,\ 18}=15.1$, $P<0.001$; Fig. 5). For *I. pini,* both JH III-treatment and feeding caused a significant increase in activity over the acetone-treated control at 16 hr (1.28±0.32 and 0.987±0.18 fold increases, respectively; ANOVA, Dunnett's method, $P<0.001$). Interestingly, feeding alone caused an increase in GPPS activity at 8 and 16 hr post treatment in male *I. confusus* (0.44±0.13 and 1.16±0.09 fold increases, respectively; ANOVA, Dunnett's method, $P<0.05$). Moreover, it was confirmed by HPLC analysis that GPP was the only short-chain isoprenyl diphospate product of these assays.

Discussion

The present results support the hypothesis that in *I. confusus*, a member of the *I. grandicollis* subgeneric group, JH III does not exclusively regulate pheromone production.

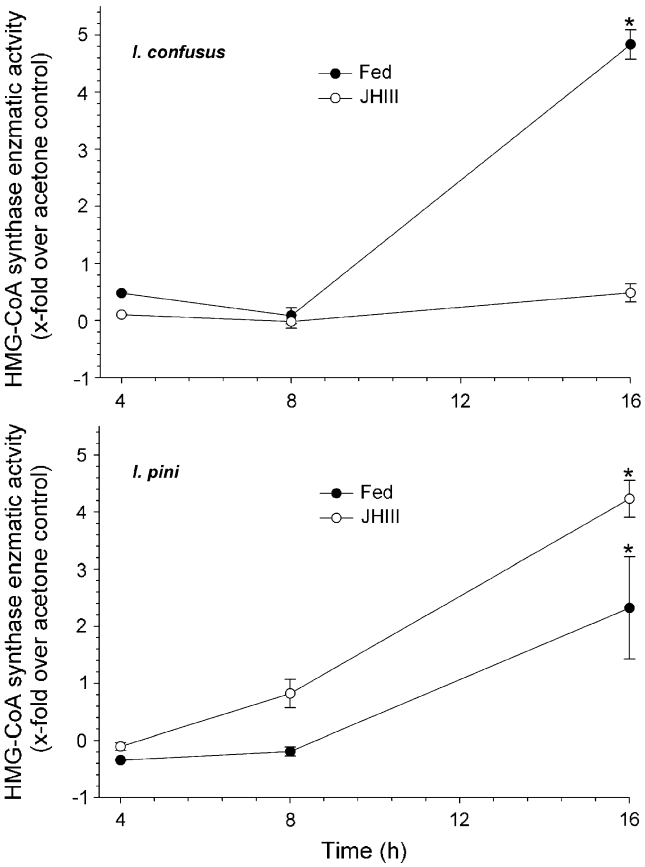

Fig. 3 Effect of feeding and JH III treatment on the enzymatic activity of HMG-CoA synthase in male *I. confusus* (top) and *I. pini* (bottom). An *asterisk* denotes a significant difference in enzyme activity relative to acetone-treated controls (Dunnett's method, $P<0.05$)

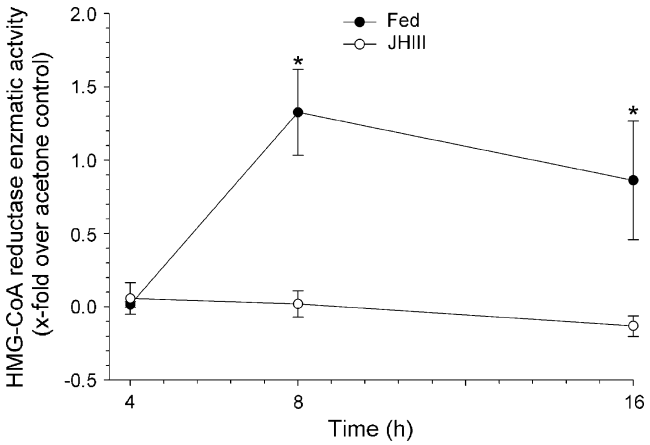

Fig. 4 Effect of feeding and JH III treatment on the enzymatic activity of HMG-CoA reductase activity in male *I. confusus*. An *asterisk* denotes a significant difference in enzyme activity relative to acetone-treated controls (Dunnett's method, $P<0.05$)

Here, we show that feeding, but not JH III treatment, stimulates pheromone production in male *I. confusus*. Previous work by Borden et al. (1969) demonstrated that gut extracts of male *I. paraconfusus* (then referred to as *I. confusus*) become highly attractive to female beetles after topical application of synthetic JH (Borden et al. 1969). However, large non-physiological doses (50–100 µg) of hormone were required to attain a strong response, and females were not attracted in bioassays to midguts treated with 10 µg of JH, the amount used in our study (Borden et al. 1969). Moreover, females were attracted to as little as 0.02 male equivalents of extract from males which had fed on host phloem, while extract from hormone-treated males elicited only a weak response (Borden et al. 1969).

The induction of mevalonate pathway genes in male bark beetles often precedes an increase in protein production, enzyme activity, and pheromone production (Tillman et al. 2004; Bearfield et al. 2006). In male *I. confusus,* either JH III treatment or feeding is sufficient to increase *HMGS*, *HMGR*, and *GPPS* transcript levels in the anterior midgut (Fig. 2). At the 4 h and 8 h time points, the transcript levels of the fed-group were slightly lower and likely due to the time necessary for JH III to be synthesized and released following feeding. At the 16 hr and 32 hr time points, however, expression levels of JH III-treated males began to decline likely due to the hormone dose being metabolized while the fed beetles showed large increases in all three genes studied (Fig. 2). Although each gene shows a modest induction of transcript at 32 hr following JH III treatment, the relative expression patterns at 32 hr post-feeding are 100 to 200-fold higher than unfed controls (Fig. 2). In addition to the transcriptional effects of JH III, these results suggest that a supplemental factor released following feeding may further induce the expression of mevalonate pathway genes in male *I. confusus*.

Feeding induces the coordinated gene expression of several earlier steps in monoterpenoid pheromone biosynthesis in both male and female *I. pini*, including *HMGS* and *HMGR*, but *GPPS,* a later step in the pathway, is induced only in males (Keeling et al. 2004). In male *I. confusus*, the differences in transcriptional regulation between JH III treatment and feeding were generally more pronounced further along the biosynthetic pathway and the disparity was most dramatic at 32 hr for all three genes (Fig. 2). The increases in relative expression with feeding were ~65-, 55-, and 120-fold above JH III-treated males for *IcHMGS*, *IcHMGR*, and *IcGPPS*, respectively (Fig. 2). Therefore, it appears that in *Ips* species, the early biosynthetic steps are more coordinately regulated by both feeding and JH III treatment than the terminal steps in the mevalonate pathway. A developmental factor may also contribute to the alternate regulation of pheromone biosynthesis due to the higher basal levels of mevalonate pathway genes. Specifically, basal *GPPS* mRNA levels in male *I. pini* are 400-500-fold above female levels (Keeling et al. 2006), suggesting that the last steps of the pathway are more

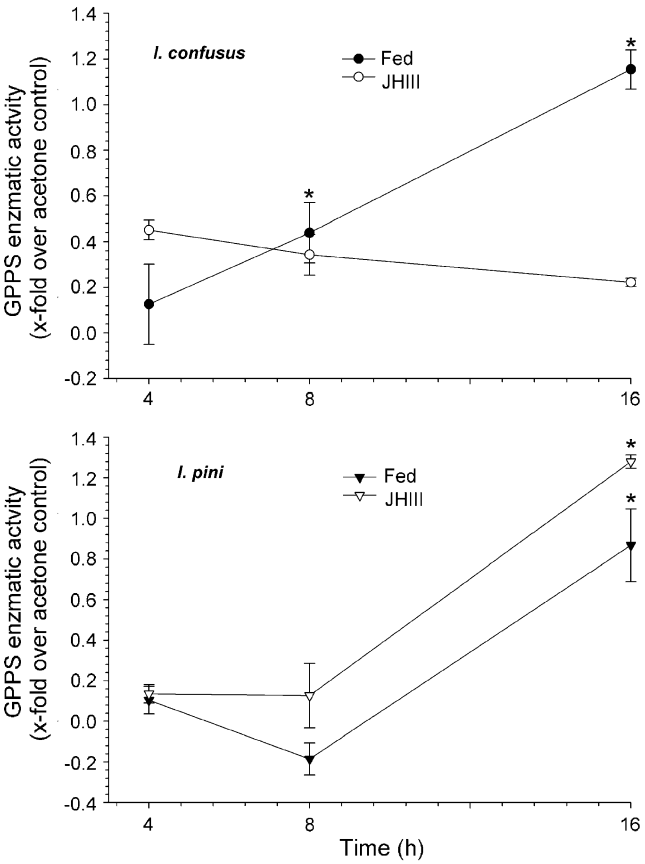

Fig. 5 Effect of feeding and JH III treatment on GPPS enzyme activity in male *I. confusus* (top) and *I. pini* (bottom). An *asterisk* denotes a significant difference in enzyme activity relative to acetone-treated controls (Dunnett's method, $P<0.05$)

developmentally regulated than *HMGS* and *HMGR*. In *I. confusus*, however, all genes studied here demonstrated only a two-fold difference in basal transcript levels (data not shown). Although not studied in as much detail, the same coordinate regulation between sexes does not exist in *D. jeffreyi* as only males demonstrate an induction of *HMGS* and *HMGR* with JH III treatment (Hall et al. 2002b; Tittiger et al. 2000, 2003). Male *D. jeffreyi* produce a mevalonate-derived aggregation pheromone component, frontalin, in response to exogenous treatment with JH III (Barkawi et al. 2003)

In *I. confusus*, feeding also caused an increase in HMGS, HMGR, and GPPS enzymatic activity in male beetles (Figs. 3, 4, and 5). Although mRNA levels were induced by JH III treatment in male *I. confusus* (Fig. 2), there was no concomitant increase in enzyme activity (Figs. 3, 4, and 5) or production of ipsdienol and ipsenol in treated insects. Similarly, the mRNA induction of *HMGR* with JH III treatment in male *I. paraconfusus*, measured by northern blot analysis, is not followed by a subsequent increase in enzymatic activity of HMGR or pheromone production (Tillman et al. 2004). In this study, we also determined that the enzymatic activity of HMGS and GPPS in *I. pini* and *I. confusus* are not uniformly regulated by JH III. Unlike *I. pini*, the activity of HMGS and GPPS in male *I. confusus* was not influenced by hormone treatment.

Tillman et al. (2004) demonstrated a difference in the regulation of pheromone production between species in the *pini* and *grandicollis* subgeneric groups. The work presented here further supports the existence of a dichotomy in the regulation of pheromone production among *Ips* species. It has also been suggested that feeding by male *I. paraconfusus* may cause the release of a brain hormone by *corpora cardiaca* which, in turn, may stimulate pheromone production (Hughes and Renwick 1977; Lu 1999). Interestingly, in preliminary experiments brain extracts from fed male *I. confusus* were sufficient to cause a modest increase in ipsenol production in JH III-treated males (unpub. data). Further study will likely reveal regulatory factors influencing pheromone biosynthesis in members of the *grandicollis* group of bark beetles.

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Comparison of Age-dependent Quantitative Changes in the Male Labial Gland Secretion of *Bombus Terrestris* and *Bombus Lucorum*

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Abstract Age-related changes of antennal-active components of male labial gland extracts were studied in two closely related bumblebee species, *Bombus terrestris* and *B. lucorum*. In *B. terrestris,* compounds eliciting electroantennogram (EAG) responses of virgin queens were ethyl dodecanoate, 2,3-dihydrofarnesal, 2,3-dihydrofarnesol, hexadecan-1-ol, octadeca-9,12,15-trien-1-ol, and geranylcitronellol. Compounds that elicited EAG responses from queens of *B. lucorum* were ethyl dodecanoate, ethyl tetradec-7-enoate, ethyl tetradec-9-enoate, ethyl hexadec-9-enoate, hexadecan-1-ol, hexadec-7-enal, octadeca-9,12-dien-1-ol, octadeca-9,12,15-trien-1-ol, and octadecan-1-ol. Quantities of these compounds in the labial glands changed significantly over the lifetime of the respective males of the two species. In both species, concentrations of the respective compounds reached their maximum within seven days after eclosion. Subsequently, a rapid decrease in the amount of EAG-active compounds occurred in *B. terrestris*, whereas in *B. lucorum* the amount of active compounds stayed approximately constant or decreased at a slow rate. Microscopy showed that in *B. terrestris* secretory cells of the labial glands undergo apoptosis from the fifth to the tenth day of life, whilst in *B. lucorum* labial gland cells remain unchanged throughout the life of the males.

Keywords Bumblebee · *Bombus terrestris* · *Bombus lucorum* · Labial gland · Male marking pheromone · Sex pheromone · GC-EAD

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Introduction

The most common European bumblebee species, *Bombus terrestris* (Linnaeus 1758) and *Bombus lucorum* (Linnaeus 1761), both belong to the subgenus *Bombus sensu stricto*. As observed for other bumblebee species during premating behavior, *B. terrestris* and *B. lucorum* males scent-mark prominent objects on their flight routes with a species-specific sex pheromone ('patrolling behavior'; Calam 1969; Schremmer 1972; Svensson 1980; Lloyd 1981; Morse 1982; Villalobos and Shelly 1987) that attracts conspecific virgin queens to the marked spots for mating (Kullenberg et al. 1970; Bergström et al. 1981; Bergman 1997).

The male-produced pheromones of these bumblebees are produced in the cephalic part of the labial gland (Kullenberg et al. 1973; Bergman and Bergström 1997). Volatile components of the gland secretion are deposited on prominent objects on the flight routes or on perches (Bergman and Bergström 1997; Kindl et al. 1999). The secretion is a complex mixture, comprised of many compounds, usually with one or two major components (Valterová and Urbanová 1997; Terzo et al. 2003). In closely related bumblebee species, the secretions may be similar but usually differ in dominant components and/or compound proportions. Although the main components of the secretion are presumed to comprise the sex pheromone, behavioral roles of specific components in female attraction have yet to be demonstrated. Thus, the actual male-produced pheromone components in secretions of bumblebee species are unknown.

The composition of bumblebee labial gland secretions is used as a tool for taxonomic identification and species and subspecies discrimination (Paterson 1993; Terzo et al. 2005; Rasmont et al. 2005; Bertsch et al. 2005; Coppée et al. 2008). However, there is great inter-individual variability in the composition of secretion components within a single species (Svensson and Bergström 1977; Ågren et al. 1979; Šobotník et al. 2008), which can make differentiation difficult, especially among related species. Recently, changes of cephalic labial gland ultrastructure in relation to age of males were reported for *B. terrestris* (Šobotník et al. 2008). It was found that the secretory activity of the gland cells is high in newly emerged males, but drops as males age. Five days after eclosion of the adult male bumblebee, the biosynthetic activity within the gland stops, and the secretory cells degenerate (Šobotník et al. 2008). Maximal gland content occurred in 2–7 day-old males, and decreased in older males. This age-related change in gland content was paralleled by the ability of gland extracts from different age males to elicit electroantennogram (EAG) responses from queen antennae. Although the study by Šobotník et al. (2008) demonstrated changes in the volume of gland content with age, there was no detailed chemical analysis of changes in the quantities of components of the secretion. They did, however, perform coupled gas chromatogram-electroantennogram detection (GC-EAD) experiments, using male labial gland secretions and queen antennae, and found at least six antennal-active compounds, ethyl dodecanoate, 2,3-dihydrofarnesal, 2,3-dihydrofarnesol, hexadecanol, octadecatrienol, and geranylcitronellol, that could potentially play a role in male sex pheromone signaling (Šobotník et al. 2008; Valterová et al. 2007).

Qualitative analysis of the labial gland extract from *B. lucorum* has previously been performed (Bergström et al. 1973; Urbanová et al. 2001). However, no attention has been paid to age of males. There are a few reports on seasonal variation in the secretion composition of other bumblebee species (Kullenberg et al. 1970; Svensson and Bergström 1977; Ågren et al. 1979), but thus far the phenomenon of age-related changes in composition has not been studied systematically.

In the present study, we report age-dependent quantitative changes of major EAG-active components of the labial gland of two related bumblebee species, *B. lucorum* and *B. terrestris*. This allowed us to determine whether the relatively time-limited labial gland secretion activity observed in *B. terrestris* also occurs in another species in the subgenus *Bombus sensu stricto.*

Methods and Materials

Insects Colonies of *B. lucorum* and *B. terrestris terrestris* (L.) were established by the two-queens cascade method (Ptáček et al. 2000). All mother queens were taken from their natural habitats during the nest-searching period in order to minimize the possible negative influence of artificial conditions on the progeny. Bumblebee colonies were kept in plastic boxes of 0.6–1 L volume and fed with honeybee pollen pellets and concentrated sugar solution (sucrose:fructose 1:1). When colonies started to produce males, male cocoons were removed from the parental hives and left to mature separately under the care of several workers supplied with food (Ptáček 1999). Freshly emerged males were removed and kept according to age. Animals of the following age were studied. *Bombus terrestris*: shortly after eclosion, 1, 2, 3, 4, 5, 7, 10, 12, 17, 20, 24, and 33 days after eclosion (five individuals of each age); *B. lucorum*: shortly after eclosion, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, and 30 days after eclosion (5–6 individuals of each age). Males were killed by freezing (−18°C) and were kept frozen prior to dissection. Labial glands were dissected and extracted with hexane (100 µl per gland) containing 1-bromodecane as internal standard (1.79 mg/ml for *B. terrestris* and 2.13 mg/ml for *B. lucorum*). Glands, in solvent, were shaken for 30 min after which the extracts were transferred to clean vials and kept at −18°C prior to analysis.

Identification of Compounds Extracts were analyzed by gas chromatography-mass spectrometry (GC-MS) using a splitless injector (200°C), mass detector (200°C, Fisons MD 800) and autosampler AI3000 (Thermo). A DB-5 ms column (30 m × 0.25 mm, film thickness 0.25 µm, Agilent Technologies) and helium gas (constant flow of 1 ml min^{-1}) were used for separations. The temperature programs differed for samples of the different species: for *B. terrestris*, 70°C (2 min. delay) to 320°C at 10°C min^{-1}; for *B. lucorum*, 70°C (2 min. delay) to 140°C at 40°C

min^{-1}, to 240°C at 2°C min^{-1}, and then to 320°C at 4°C min^{-1}. Compounds were identified based on their mass spectra and on co-chromatography with synthetic or commercially available standards.

Gas chromatography-electroantennogram detection GC-EAD experiments were performed with a 5890A Hewlett-Packard gas chromatograph (GC) equipped with a DB-5 column (30 m×0.25 mm, film thickness 0.25 µm, J & W Scientific). The column was split by a Graphpack 3D/2 four-arm splitter. The splitter led the eluate to flame ionization (FID) and EAD detectors. N_2 make-up gas at 20 ml min^{-1} was introduced via one arm of the splitter. Labial gland extracts (1–5 µl) were injected in the splitless mode. The GC was temperature programmed from 50°C (2 min. delay) to 270°C at 30°C min^{-1}. The temperature of the injector and FID were set to 230 and 260°C, respectively. The EAD detector consisted of a queen antenna connected via two glass Ag/AgCl electrodes to a universal AC/DC 10XProbe (Syntech, Hilversum, The Netherlands). The EAD and FID signals were fed to a computer via the serial IDAC interface box (Syntech) and analyzed by using GC-EAD software (Syntech). The antenna was exposed to compounds that eluted from the GC via an Effluent Conditioner Tube (Syntech) heated to 180°C. Virgin queens (N=4) used for GC-EAD recording were kept at low temperature (5°C) and high humidity until use. Isolated antennae, with the distal tips excised, were used for recordings. Each antenna was used only once.

Chemicals The following standards were used for the quantification of EAG-active components of the labial gland secretions: (*E*)-farnesol (Firmenich, Geneva, Switzerland), geranylgeraniol (ICN, Irvine, CA, USA), ethyl tetradec-9-enoate (Nu-Check-Prep, Elysian, MN, USA). (*Z,Z,Z*)-Octadeca-9,12,15-trien-1-ol, ethyl dodecanoate, hexadecan-1-ol, octadecan-1-ol, (*Z,Z*)-octadeca-9,12-dien-1-ol, and ethyl hexadec-9-enoate were purchased from Sigma (St Louis, MO, USA). (*Z*)-Hexadec-9-enal was prepared earlier in our laboratory.

Quantitative Analyses Only EAD-active compounds were quantified. For *B. terrestris*, 2,3-dihydrofarnesol, 2,3-dihydrofarnesal, geranylgeraniol, geranylcitronellol, (*Z,Z,Z*)-octadeca-9,12,15-trien-1-ol, and ethyl dodecanoate were quantified; *B. lucorum:* ethyl dodecanoate, ethyl tetradec-7-enoate, ethyl tetradec-9-enoate, ethyl hexadec-9-enoate, hexadecanol, hexadec-7-enal, octadecan-9,12-dienol, octadecan-9,12,15-trienol, and octadecanol were quantified. Quantification was carried out in Total Ion Current mode of the mass detector and based on peak areas. Because the different compounds were present in samples at widely differing concentrations, we had to avoid overloading the mass detector with the most abundant component. Separation of ethyl tetradec-7-enoate and ethyl tetradec-9-enoate under the conditions used was poor. Therefore, these two isomers were quantified together according to the external calibration for ethyl tetradec-9-enoate. Previous studies (Urbanová et al. 2001) showed that the concentration ratio of these isomers in the labial gland extracts was: $\mathrm{area}_{\mathrm{ethyltetradec-9-enoate}} / \mathrm{area}_{\mathrm{ethyltetradec-7-enoate}} = 187.5$. We assumed that the difference in responses of these isomers in the mass detector was less than 5%, and the concentration ratio remained constant over a bumblebee's life.

The calibration curve for (*Z*)-hexadec-7-enal was constructed by using (*Z*)-hexadec-9-enal, as the correct isomer was not available. 2,3-Dihydrofarnesol and 2,3-dihydrofarnesal quantification was calibrated by using farnesol, and geranylcitronellol was calbrated by using geranylgeraniol, due to unavailability of standards. Considering the close similarities in structures between calibrants and compounds, we assumed the quantification error to be low. Octadeca-9,12-dienol and octadeca-9,12,15-trienol could not be separated, but the same approach and assumption as used for ethyl tetradec-7-enoate and ethyl tetradec-9-enoate were used for the samples and quantitative calibration.

The same internal standard (IS; 1-bromodecane, ~2 mg/ml) was used for both calibration and sample sets. Calibration curves were constructed from peak area (ratios of standard/IS). Second degree polynomial equations were used to fit the obtained data. Unknown sample concentrations were calculated by using the calibration curve equations and expressed in µg per gland for each component. Mean values and standard errors were calculated for each male age group.

Microscopy Fixed cephalic parts of labial glands of *B. lucorum* originated from males of ages, pharate imago (darkly colored males enclosed in pupal cuticle shortly before eclosion), <1 d, 1, 2, 3, 4, 5, 6, 9, and 13 days. Two to four samples of each age were studied using optical microscopy and, as only slight differences were observed over the adult life of a male, only a single sample of each age was studied by transmission electron microscopy. Dissection took place in a droplet of fixative (2% glutaraldehyde and 2.5% formaldehyde in 0.1 M phosphate buffer), in which the tissue stayed for 1 day at ambient temperature. After post-fixation in 1.5% OsO_4 in 0.1 M phosphate buffer, the samples were dehydrated by passing through a series of ethanol-water mixtures (50, 75, and finally 100%). Tissues were then embedded into standard Spurr resin. Semithin sections (1 µm) were stained with Azure II and observed under Amplival (Zeiss) optical microscope (equipped with Canon EOS 300D camera). Ultrathin sections were studied with a Jeol 1011 transmission electron microscope.

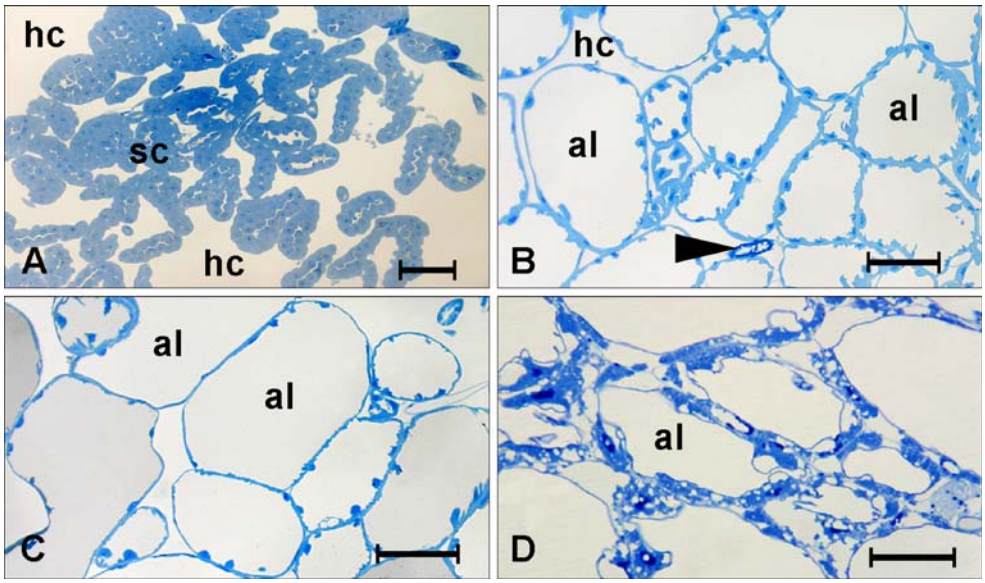

Fig. 1 Development of acini in the cephalic part of the labial gland of *Bombus lucorum* (A–C) and *B. terrestris* (D) (optical microscopy); **a** development of acini in a 1-day-old adult male of *B. lucorum*; **b** development of acini in a 3-day-old male of *B. lucorum*. The arrowhead marks an excretory duct of the labial gland; **c** development of acini in a 13-day-old male of *B. lucorum*; **d** development of acini in a 13-day-old male of *B. terrestris* (all cells are already dead, but the acini are still full of secretion). Bar represents 100 µm in all figures. Abbreviations: al, acinar lumen; hc, haemocoel; sc, secretory cells

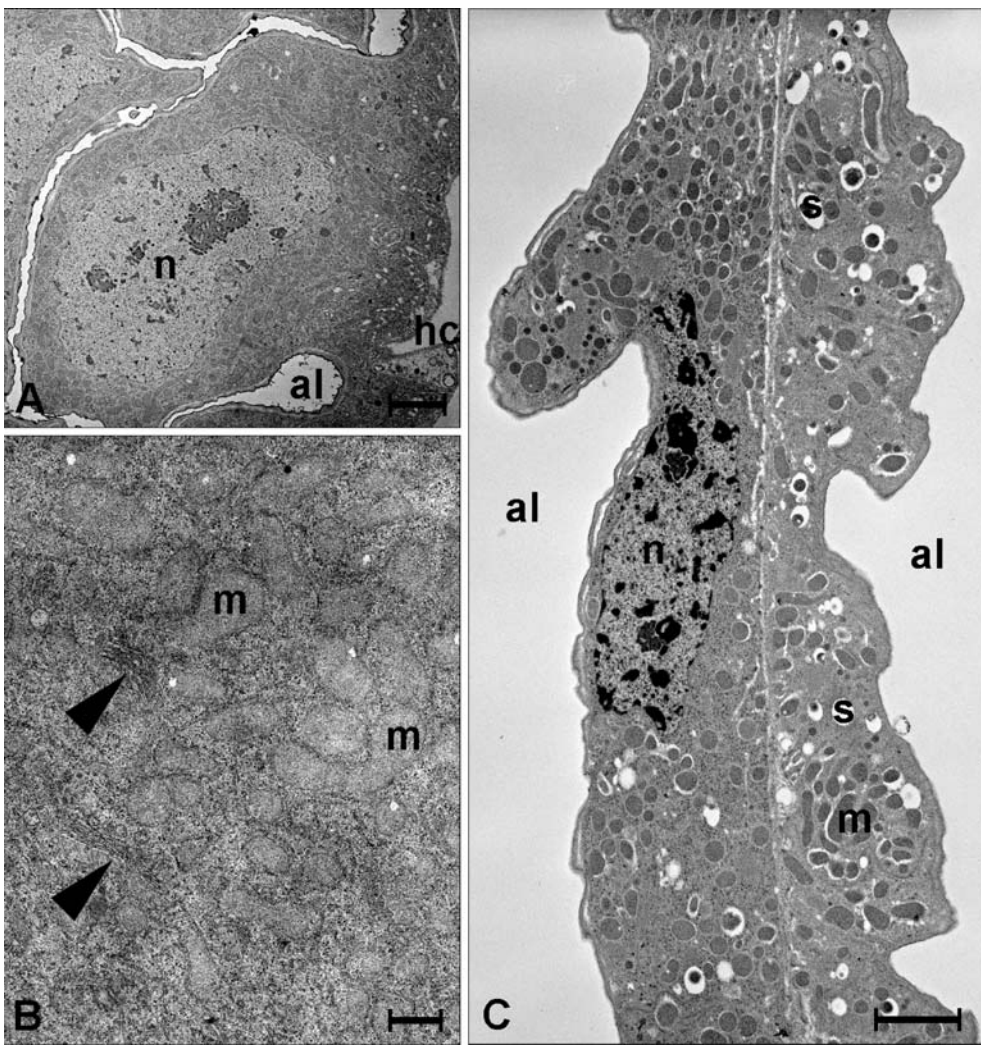

Fig. 2 Transmission electron micrographs of acinar secretory cells in *Bombus lucorum*; **a** whole secretory cell in pharate male imago. Note the active transport at the cell base occurring as pinocytotic vesicles. Bar represents 2 µm; **b** detail of cytoplasm in <1d adult male. Arrowheads mark smooth endoplasmic reticulum producing a secretion. Bar represents 500 nm; **c** walls of two neighbouring acini in a 13-day old male. Bar represents 2 µm. Abbreviations: al, acinar lumen; hc, hemocoel; m, mitochondria; n, nucleus; s, secretion within the cells

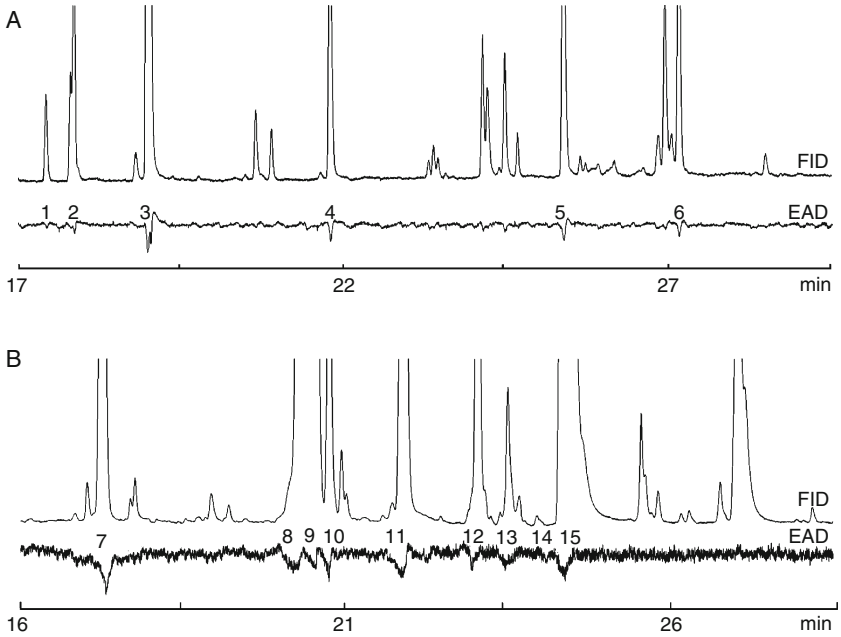

Fig. 3 Coupled gas chromatograph-electroantennogram detection recording of the labial gland extract of 7-day-old bumblebee males. **a** *Bombus terrestris*; active compounds: **1** = ethyl dodecanoate; **2** = 2,3-dihydrofarnesal; **3** = 2,3-dihydrofarnesol; **4** = hexadecan-1-ol; **5** = octadeca-9,12,15-trien-1-ol; **6** = geranylcitronellol. **b** *B. lucorum*; active compounds: **7** = ethyl dodecanoate, **8** = ethyl tetradec-7-enoate, **9** = ethyl tetradec-9-enoate, **10** = hexadec-7-enal; **11** = ethyl hexadec-9-enoate; **12** = hexadecan-1-ol; **13** = octadeca-9,12-dien-1-ol; **14** = octadeca-9,12,15-trien-1-ol; **15** = octadecan-1-ol. FID (upper trace) = flame ionixation detection, EAD (lower trace) = electroantennogram detection

Results

Gland Microscopy Microscopy of the cephalic labial gland showed differences between the two species. Unlike *B. terrestris*, the labial gland of *B. lucorum* remained functional throughout the life of the adult male. Additionally, in *B. lucorum*, the production of lipid secretion by the smooth endoplasmic reticulum (SER) started earlier (at time of emergence) (c.f., in *B. terrestris* production started on the 2nd day after emergence). The lipids were continuously secreted at the cell apices and, as their volume increased, were accompanied by swelling of the lumen and a decrease in cell layer thickness (Fig. 1a–c; optical microscopy).

The active transport of precursors from the hemolymph was observed in young males of both species, with the transport stopping during the third day after eclosion in *B. terrestris* (Šobotník et al. 2008), but continuing in males of *B. lucorum* up to the 13th day after eclosion. This

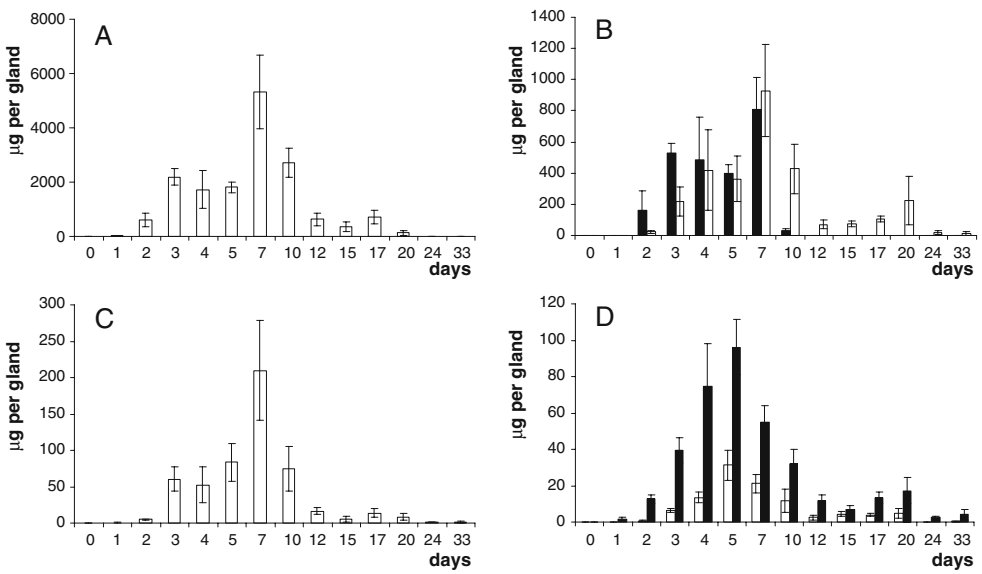

Fig. 4 Concentration changes, with regard to age of adult male, of electroantennogram-active compounds in the cephalic labial gland of *Bombus terrestris* males (mean ± standard error). **a** 2,3-dihydrofarnesol; **b** geranylcitronellol (white bars) and ethyl dodecanoate (black bars); **c** hexadecan-1-ol; **d** 2,3-dihydrofarnesal (white bars) and octadeca-9,12,15-trien-1-ol (black bars)

difference is associated with the different fates of the secretory cells in both species. The cells died after several days of secretory activity in *B. terrestris* (between the 5th and 10th day, Fig. 1d), whereas they conitnued to produce secretion throughout the life of adult *B. lucorum* males. However, even in older *B. lucorum* males, flattening of cells, lower volume of SER, and fewer droplets of secretion in the cell cytoplasm were observed (Fig. 2a,b; electron microscopy) indicating a decreasing rate of secretion production.

GC-MS, GC-EAD, and quantitative analyses In the labial gland of *B. terrestris,* the EAD-active compounds were identified as ethyl dodecanoate, 2,3-dihydrofarnesal, 2,3-dihydrofarnesol, hexadecanol, octadeca-9,12,15-trienol, and geranylcitronellol (Fig. 3a). In *B. lucorum*, ethyl dodecanoate, ethyl tetradec-7-enoate, ethyl tetradec-9-enoate, ethyl hexadec-9-enoate, hexadecanol, hexadec-7-enal, octadeca-9,12-dienol, octadeca-9,12,15-trienol, and octadecanol elicited antennal responses in conspecific queens (Fig. 3b).

The EAD-active compounds of both species were quantified. In *B. terrestris*, the most abundant EAD-active component of the extract was 2,3-dihydrofarnesol (Fig. 4a). Males produced small quantities (ca. 20 µg/gland) of this compound on the day of eclosion. The quantity of this compound increased up to 2 mg/gland over the next two days, and remained roughly constant at this level for three more days. On the 6th and 7th days following eclosion, the concentration increased almost three times to ca. 6 mg/gland. Subsequent to this, the amount of 2,3-dihydrofarnesol decreased dramatically, such that at 10 days after eclosion, it fell to ca. 0.5 mg/gland. By day 20, 2,3-dihydrofarnesol was present in the gland at a very low amount (2 µg/gland). A similar pattern was observed for geranylcitronellol (1 µg–1 mg/gland, Fig. 4b) and hexadecanol (0.5 µg–200 µg/gland, Fig. 4c). A different age-dependent pattern was observed for ethyl dodecanoate, which appeared in the secretion later than the terpenes (at 2 days, 0.5 µg/gland), increased to its maximum concentration (800 µg/gland) on day 7, and then dropped to 0.7 µg/gland by day 20 (Fig. 4b). The remaining EAD-active compounds, octadecatrienol and 2,3-dihydrofarnesal, reached a maximum concentration around day five (100 µg/gland and 30 µg/gland, respectively, Fig. 4d).

The concentration profiles of the most abundant EAD-active compounds (i.e., ethyl tetradec-7-enoate and ethyl tetradec-9-enoate) present in the gland of *B. lucorum*, (Fig. 5a), differed from those in *B. terrestris.* In *B. lucorum*, no significant maxima were observed in the profile of the most abudnant EAD-active compounds, with the quantities remaining relatively constant over the adult life of the males. Ethyl tetradec-7-enoate and ethyl tetradec-9-enoate were both present (ca 0.9 mg/gland) in the gland on the day after eclosion, with maximum quantities (total of 1.5 mg/gland) occurring between days 7–20. The quantities of these two compounds declined significantly by day 25. Other EAD-active compounds, ethyl dodecanoate and hexadecanol (Fig. 5b), were present at roughly one tenth the amount (113 and 91 µg/gland, respectively) of the main component, but their concentration profiles exhibited a similar pattern with no significant maximum or changes between days 4–10. However, by day 15, the amount of hexadecanol increased, especially in comparison to ethyl dodecanoate, and reached a maximum in 25-day old males (185 µg/gland). Patterns in concentration changes similar to that of hexadecanol were observed for ethyl hexadec-9-enoate and hexadec-7-enal (Fig. 5c). The alcohols octadeca-9,12-dienol, octadeca-9,12,15-trienol (Fig. 5d),

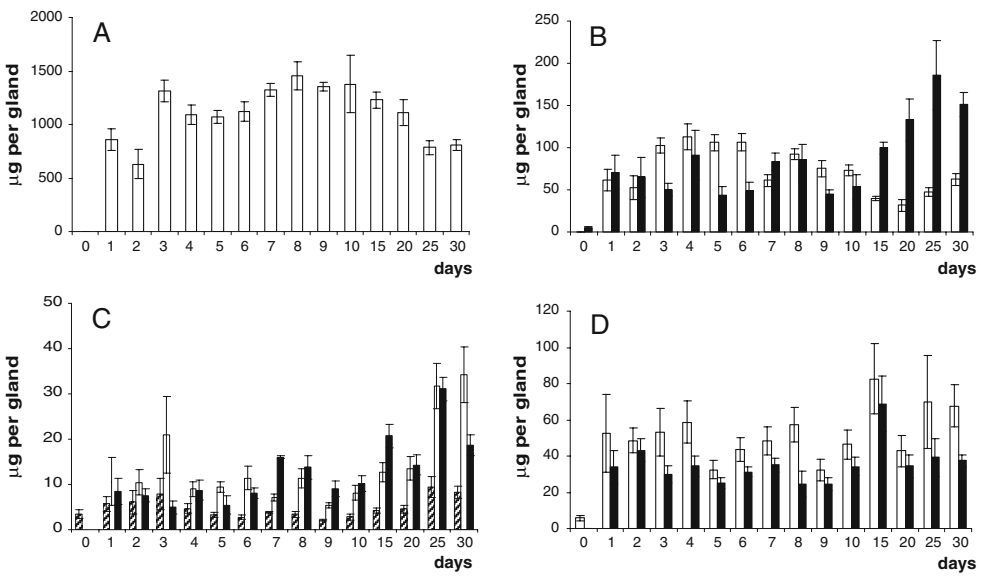

Fig. 5 Concentration changes, with regard to age of adult male, of electroantennogram-active compounds in the cephalic labial gland of *Bombus lucorum* males (mean ± standard error). **a** total ethyl tetradec-7-enoate and ethyl tetradec-9-enoate; **b** ethyl dodecanoate (white bars) and hexadecan-1-ol (black bars); **c** octadecan-1-ol (hatched bars), ethyl hexadec-9-enoate (white bars), and hexadec-7-enal (black bars); **d** octadeca-9,12-dien-1-ol (white bars) and octadeca-9,12,15-trien-1-ol (black bars)

and octadecanol (Fig. 5c) remained at a similar concentration (30–70 μg/gland) throughout the life of the male.

Discussion

The patterns in the quantitative profiles of the various EAD-active compounds corresponded well with the physiological and morphological changes observed in the labial glands for both species. While in *B. lucorum*, the secretory activity of the acinar cells continued over the lifetime of adult males, in *B. terrestris*, production stopped in males older than 10 days. The reasons behind the profound differences in labial gland morphlogy and chemical production between the closely related species are not clear and cannot be explained by lifetime of males, since laboratory observations show no difference in longevity (~50 days) of both species.

We speculate that the age-dependent changes in gland content may play a role in mate selection. Maximal labial gland content (and activity of secretory cells) was found in 3–5 day-old males of *B. terrestris*, which corresponds well with the time when males usually leave their natal nests (fifth day after eclosion) and start to mark and patrol. Additionally, maximal content in the gland matches maximal sperm content (6th day post-emergence; Tasei et al. 1998). In the laboratory, *B. terrestris* males mate between day 6–27 with the probability of successful mating dropping dramatically after day 11 (Tasei et al. 1998). Information about optimal reproductive behavior and physiology in *B. lucorum* is not available, but it would be interesting to know whether it matches the greater duration of labial gland content of EAD-active compounds found in this study.

It has been hypothesized that virgin queen bumblebees searching for a mate are likely to be more attracted to locations where higher amounts of secretion have been deposited by patrolling males (Ågren et al. 1979). Thus, given our results, it is possible that younger males of *B. terrestris* may have an advantage, over older males, in attracting queens, because their scent marks are likely to contain higher amounts of labial gland secretions than those of older males. Such an advantage would not be expectecd in *B. lucorum*, since young and old males have similar quantities of labial gland secretions.

Earlier reports on the variation in composition of the labial gland secretions of bumblebees are scarce. Kullenberg et al. (1970) described considerable seasonal variation in the diterpene content in the glands of *B. hortorum* and *B. hypnorum*. Svensson and Bergström (1977) found that diterpenic components in the labial gland secretion of *B. pratorum* males appear later in the season. Ågren et al. (1979) studied changes in labial gland secretion of various bumblebees species. No labial gland secretion compounds were observed in pupae of *B. hypnorum*. The compounds appeared from day 1 after emergence, and their amounts increased rapidly over the next 4–7 days. For *B. lapidarius*, measurements of gland content were not made until 7 days after eclosion. Secretion content had dropped 14 days after eclosion and further declined to undetectable by 19 –29 days. A similar declining trend was observed for *B. hortorum* males (Ågren et al. 1979). Although this study utilized an analytical technique (TLC) that did not allow precise quantifications of compounds to be made, the results are consistent with our observations for *B. terrestris*.

Although attractiveness of the male labial gland secretion for young queens has been demonstrated by Bergman (1997), roles of individual components in the blend have not been studied. Our study on the quantitative changes of EAG-active chemicals has identified a number of potential pheromone components, which should be tested in bioassays.

Finally, our results show that the production and composition of the labial gland secretion of male bumblebees vary considerably with age. Thus, chemotaxonomic studies that use labial gland secretions to distinguish between closely related species should take this variation into account, perhaps by analyzing solely mature and sexually active males. Such chemotaxonomic studies are best combined with other techniques such as morphology and genetic analysis, for differentiating clsoely related species (Bertsch et al. 2005).

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Identification of Female-produced Sex Pheromone of the Honey Locust Gall Midge, *Dasineura gleditchiae*

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Abstract The honey locust gall midge, *Dasineura gleditchiae* Osten Sacken 1866 (Diptera: Cecidomyiidae) is the main pest of ornamental varieties of the honey locust tree, *Gleditsia triacanthos* L., in North America, and is now becoming a pest of concern in Europe. Female midges were observed to emerge in the early morning with their ovipositor extended until they mated. Volatiles were collected from virgin females in a closed-loop stripping apparatus and analyzed by gas chromatography (GC) coupled to electroantennographic (EAG) recording from the antenna of a male midge. A single EAG response was observed, which was assumed to be to the major component of the female sex pheromone. This was identified as (*Z*)-2-acetoxy-8-heptadecene by comparison of its mass spectrum and GC retention times on different columns with those of synthetic standards and by micro-analytical reactions. This compound was synthesized, and the individual enantiomers were produced by kinetic resolution with lipase from *Candida antarctica*. Analysis of the naturally-produced compound on a cyclodextrin GC column indicated it was the *(R)*-enantiomer. In EAG dose-response measurements, the *(R)*-enantiomer alone or in the racemic mixture evoked significant responses from the antennae of male *D. gleditchiae*, whereas the *(S)*-enantiomer did not. In field trapping tests, the *(R)*-enantiomer attracted male *D. gleditchiae.* The racemic compound was equally attractive, but the *(S)*-enantiomer was not attractive. Both the pure *(R)*-enantiomer or racemic (*Z*)-2-acetoxy-8-heptadecene, applied to red rubber septa in a dose range of 3–30 μg, constitute a strongly attractive bait in sticky traps for monitoring the flight of *D. gleditchiae.*

Keywords Honey locust gall midge · *Dasineura gleditchiae* · Cecidomyiidae · Chiral sex pheromone · Enantiomers · (*Z*)-2-acetoxy-8-heptadecene · (2*R*,8*Z*)-2-acetoxy-8-heptadecene · *Gleditsia triacanthos* · Ornamental tree · Diptera

Introduction

The honey locust gall midge, *Dasineura gleditchiae* Osten, Sacken 1866 (Diptera: Cecidomyiidae), already known as the most important pest of honey locust tree, *Gleditsia triacanthos* L., in North America (Thompson et al. 1998), has now appeared in several European countries, e.g., the Netherlands (Nijveldt 1980), Italy (Bolchi and Volonte 1985), Great Britain (Halstead 1992), Switzerland (Fischer and Pivot 1992), Serbia (Simova-Tossic and Skuhravá 1995), and Hungary (Ripka 1996). Soon after its appearance in Hungary, it severely damaged the aesthetic value of thornless, *inermis* cultivars of *G. triacanthos*, which are favoured ornamental trees in urban green areas, and public concern prompted the search for control methods. The developing larvae are hidden and protected inside the galls. Morever, only a few pesticides are registered for application in urban areas, and their use is restricted, such that often only a single treatment is permitted. These constraints underline the importance of the exact timing of application

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of control measures, for which pheromone traps could be suitable tools. Monitoring the flight by means of pheromone-based trapping would be particularly advantageous in view of the sharply-synchronized, extremely short flight periods, typically lasting no longer than a few days, and of the development of several subsequent generations in the course of a season.

Here, we report the identification of the female-emitted sex pheromone of *D. gleditchiae* and field trapping tests with the synthetic pheromone.

Methods and Materials

Insects Insects were collected as mature larvae or pupae inside galls on infested leaves of *G. triacathos* "Sunburst" trees located in Budapest, Hungary. Infested leaves were placed in cylindrical glass containers (15 cm diam, 20 cm high) at room temperature under 18:6 hL:D photoperiod at a relative humidity 80–90%. Emerging adults were collected daily and separated by sex on the basis of the morphological differences in their antennae (Skuhravá 1997). Females mate in the morning hours of the day of emergence, lay eggs on the same day, and die soon afterwards. Thus, midges were collected early in the morning, and were assumed to be unmated.

Calling, Diel Dependence and Length of Mating Freshly-emerged adults were used for the observations, and tests were conducted in a greenhouse in Budapest under ambient conditions with one wall open but sheltered from direct sunlight. A single female was placed in a Petri-dish (20 cm diam) containing a freshly cut leaflet of *G. triacanthos* "Sunburst", and allowed to acclimatize for a few minutes. Then, 5 males were introduced into the Petri dish (25 replicates). Copulatory behavior was observed, and the duration of copula measured. For statistical analyses, a *t*-test was used.

Collection of Volatiles Freshly-emerged, unmated females were transferred from the emergence chambers into the glass tube (125 ml) of a closed-loop stripping apparatus (CLSA) (Brechbühler AG, Schlieren, Switzerland), equipped with a MB-21E metal bellows vacuum pump (Metal Bellow Corporation, Sharon, MA, USA) and collection filter containing activated charcoal (1.5 mg). Collections were run from 9.00 h to 14.00 h to coincide with the mating period in the experimental greenhouse where the observations of mating were performed. Collections were conducted over 3 consecutive days, and dead females were replaced daily by fresh ones. Two such collections were made using totals of ca. 400 and 1,800 females, respectively. Trapped volatiles were eluted from the charcoal filter with hexane (40 µl; "ProAnalysi", Merck, Darmstadt, Germany).

Electroantennography (EAG) EAG responses were recorded with glass electrodes (1.17 mm i.d.; Syntech, Hilversum, The Netherlands) pulled to a fine point and filled with Ringer solution (Beadle and Ephrussi 1936). These were attached to silver/silver chloride electrodes held in MP15 micromanipulators and connected to an IDAC 232 amplifier (Syntech). The reference electrode was inserted into the back of the isolated head of a male *D. gleditchiae*, and the intact tip of one antenna was inserted into the recording electrode, which was cut to give a close fit. The required amounts of synthetic or natural pheromone in hexane (10 µl) were applied to filter paper (1 × 1 cm). The natural pheromone was quantified by gas chromatography with flame ionization detection (GC-FID), based on comparison of the area of the peak on the GC trace corresponding to the EAG-active component with those of standard acetates. The amount of the racemic pheromone was doubled with respect to the single enantiomers at each dose level, so that the amounts of each enantiomer would correspond to amounts of the individual enantiomers tested. Stimuli were delivered by puffing air (1 ml) through a Pasteur pipette that contained the filter paper into a moistened air stream (660 ml/min) directed towards the antennae. The series of stimuli was tested on 5 antennal preparations. For statistical analysis, responses to synthetic samples were normalized to the response evoked by an aliquot of volatile collection containing ca. 2.8 ng of natural pheromone, corresponding to ca. 150 female equivalent hours (FEH). Data were transformed to log(x+1), and subjected to ANOVA. If the *F* value was significant, differences between means were tested for significance by the Games-Howell *post-hoc* test, using the program SuperANOVA® (Abacus Concepts Inc., Berkeley, CA, USA).

For gas chromatographic analyses with simultaneous electroantennographic detection (GC-EAD), the above EAG was linked by an effluent conditioning assembly (Syntech) to a 6890 N GC (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a DB-Wax column (30 m × 0.32 mm × 0.25 µm film thickness; J&W Scientific). Injections were made in splitless mode (220°C). The oven temperature was held at 60ºC for 1 min, then programmed at 10ºC/min to 220ºC and held for 20 min. The carrier gas was helium (4.0 ml/min).

Gas Chromatography Linked to Mass Spectrometry (GC-MS) GC-MS analyses were carried out on a CP 3800 gas chromatograph linked to a Saturn 2200 ion trap mass spectrometer (Varian, Oxford, UK) operated in electron impact mode. Samples were analyzed with fused capillary columns (30 m × 0.25 mm i.d. × 0.25 µm film thickness) coated with polar (Supelcowax-10, Supelco, Gillingham, Dorset, UK) or non-polar (VF5, Varian) phases. Oven temperature was held at 50°C for 2 min, then programmed at 6°C/min to 250°C

and held for 5 min. Helium was used as the carrier gas (1.0 ml/min), and injection was splitless (200°C). Retention times were converted to retention indices relative to the retention times of *n*–alkanes or acetates (Zellner et al. 2008).

Enantioselective Gas Chromatography For analytical separation of enantiomers, an HP6890 gas chromatograph was used, fitted with a fused silica capillary column (30 m × 0.32 mm i.d.) coated with CP-Chirasil-DexCB (Varian) and FID (250°C). Carrier gas was helium (2.4 ml/min), injection was splitless (200°C), and the oven temperature was held at 60°C for 2 min, then programmed at 10°C/min to 170°C.

Microanalytical Reactions For hydrogenation, 10% palladium on charcoal catalyst was washed twice with hexane, and then one drop of the suspension was added to a collection of volatiles (5 µl) from female *D. gleditchiae* in hexane. Hydrogen was bubbled into the suspension for 2 min through a length of deactivated fused silica tubing (20 cm, 0.32 mm i.d.), and the product was analyzed by GC-MS on the polar column.

For reaction with dimethyl disulfide (DMDS), an aliquot (5 µl) of a collection of volatiles from female *D. gleditchiae* containing approximately 25 ng of the EAG-active component was mixed with DMDS (10 µl; SigmaAldrich, Gillingham, Dorset, UK) and a solution of iodine in diethyl ether (2 µl, 0.1%) (Leonhardt and DeVilbiss 1985). After standing 6 h at 40°C, the reaction mixture was treated with hexane (100 µl) and aqueous sodium thiosulfate solution (20 µl, 5%), and the layers were mixed until the iodine color had disappeared. The hexane layer was removed, dried with magnesium sulfate, and analyzed by GC-MS on the non-polar column.

Synthesis Nuclear Magnetic Resonance (NMR) spectra were recorded in $CDCl_3$ on a JEOL EX270 machine at 270 MHz for ^1H and 67.8 MHz for ^{13}C. Infrared (IR) spectra were recorded as thin films with a Perkin Elmer 881 grating spectrophotometer (Perkin Elmer, Beaconsfield, Bucks., UK).

(*Z*)-2-Acetoxy-8-heptadecene was synthesized by addition of methylmagnesium iodide to (*Z*)-7-hexadecenal followed by acetylation of the resulting alcohol (Fig. 1). The racemic material was resolved by hydrolysis with lipase from *Candida antarctica* that gave unreacted (2*S*)-acetate and the (2*R*)-alcohol, which was acetylated to give the corresponding acetate (Fig. 1).

Thus, a sample of (*Z*)-7-hexadecenyl acetate prepared previously at Natural Resources Institute, University of Greenwich, Chatham Maritime, Kent, UK, (NRI) by a standard acetylenic route was hydrolyzed with potassium carbonate in methanol, and the resulting alcohol was oxidized to the aldehyde with pyridinium chlorochromate in dichloromethane (Corey and Suggs 1975). Methyl magnesium iodide was prepared by addition of methyl iodide (2.84 g, 20 mM) in dry diethyl ether (10 ml) to magnesium turnings (0.6 g, 25 mM) in diethyl ether (20 mM) under nitrogen.

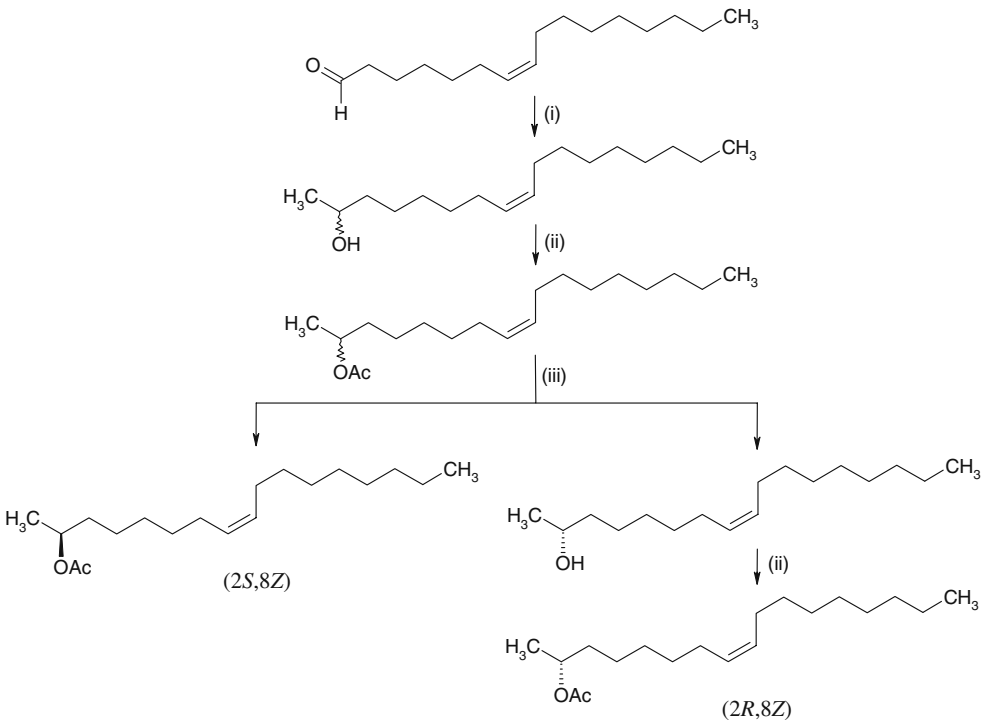

Fig. 1 Synthesis and resolution of (*Z*)-2-acetoxy-8-heptadecene (reagents: (i) MeMgI in diethyl ether; (ii) acetic anhydride/pyridine; (iii) lipase from *Candida antarctica* in phosphate buffer pH 7)

Titration indicated the resulting solution was 0.76 M. To this solution (7.5 ml, 6 mM) was added (*Z*)-7-hexadecenal (0.714 g, 3 mM) in diethyl ether (10 ml) dropwise with stirring and ice cooling. Standard aqueous workup and Kugelrohr distillation gave (*Z*)-8-heptadecen-2-ol (b.p. 140°C, 0.04 mm Hg; 0.7 g, 93%). IR (film): ν 3420 cm^{-1} (O-H); MS: *m/z* (%) 254 (M^+, 6), 236 (M-H_2O, 3), 138 (8), 137 (7), 125 (11), 124 (11), 111 (8), 110 (27), 109 (31), 97 (26), 96 (64), 95 (60), 83 (22), 82 (52), 81 (100), 69 (23), 68 (21), 67 (100), 57 (12), 55 (38), 54 (12), 45 (29), 43 (21), 41 (38).

The alcohol (0.254 g, 1 mM) was acetylated with acetic anhydride (0.2 ml) in pyridine (0.2 ml) for 5 h at room temperature. Standard aqueous workup and Kugelrohr distillation gave (*Z*)-2-acetoxy-8-heptadecene containing approximately 5% of the *E* isomer (b.p. 160°C/ 0.04 mm; 0.25 g, 85%). IR (film): ν 1735 cm^{-1} (C=O); ^{1}HNMR: δ 0.88 (t, *J* = 6.8 Hz, 3H, C$\underline{H}_3$-CH_2), 1.20 (d, *J* = 6.2 Hz, 3H, C$\underline{H}_3$-CH), 1.2–1.4 (br m, 20H, -C$\underline{H}_2$-), 1.95–2.05 (br m, 4H, C$\underline{H}_2$-CH=), 2.02 (s, 3H, C$\underline{H}_3$-C=O), 4.88 (hex, J = 6.2 Hz, 1H, CH_3-C$\underline{H}$-O), 5.3–5.4 (m, 2H, C=C$\underline{H}$); ^{13}C NMR: δ 14.12, 19.97, 21.39, 22,69, 25.32, 27.11, 27.24, 29.11, 29.34, 29.54, 29.63, 29.78, 31.92, 35.92, 71.07, 129.63, 130.11, 170.78.

(Z)-2-Acetoxy-8-heptadecene (0.16 g, 0.54 mM) was stirred with dipotassium hydrogenphosphate buffer (0.1 M, pH 7; 3 ml) and lipase acrylic resin from *Candida antarctica* (Sigma-Aldrich; 25 mg) overnight at room temperature. The mixture was extracted twice with 10% diethyl ether in petroleum ether (10 ml), and the extracts were washed once with brine (10 ml) and dried over magnesium sulfate. After removal of solvent on a rotary evaporator, the residue (0.15 gm) was chromatographed on silica gel (7 g) eluted with a gradient of diethyl ether in petroleum ether from 2% to 20%. The unreacted acetate fraction contained (2*S*,8*Z*)-2-acetoxy-8-heptadecene (70 mg) with enantiomeric excess (ee) of 99.0%. The alcohol fraction was acetylated with acetic anhydride (0.1 ml) in pyridine (0.1 ml) at room temperature overnight. Standard aqueous workup gave (2*R*,8*Z*)-2-acetoxy-8-heptadecene (70 mg) with 99.1% ee.

2-Acetoxyheptadecane was prepared from 2-heptadecanone synthesized previously at NRI by addition of methyl lithium to hexadecanoic acid in ether (Jorgenson 1970). The ketone was reduced to 2-heptadecanol with sodium borohydride in aqueous ethanol and acetylated with acetic anhydride in pyridine (b.p. 140°C/0.04 mm Hg). IR (film): ν 1735 cm^{-1}; MS: *m/z* (%) 255 (1), 254 (1), 180 (2), 166 (2), 152 (4), 139 (4), 138 (4), 125 (13), 124 (8), 111 (35), 110 (11), 98 (8), 97 (53), 96 (16), 95 (8), 85 (12), 84 (9), 83 (41), 82 (15), 81 (13), 71 (17), 70 (18), 69 (39), 67 (16), 61 (7), 57 (28), 56 (15), 55 (40), 43 (100), 42 (7), 41 (38).

All other positional isomers of acetoxyheptadecane were synthesized by reduction and acetylation of the corresponding heptadecanones, which were available commercially or were synthesized by addition of an organolithium to a carboxylic acid (Jorgenson 1970). (2*S*,12*Z*)-2-Acetoxy-12-heptadecene, the pheromone of the pistachio twig borer, *Kermania pistaciella* (Gries et al. 2006), was kindly provided by Prof. Gerhard Gries.

Field Trapping Tests Synthetic compounds were applied to red rubber dispensers (MSZ 9691/6, TAURUS, Budapest, Hungary) in hexane (20 μl). Delta-shaped traps with transparent walls (Csalomon® RAG, Plant Protection Institute Hungarian Academy of Sciences, Budapest, Hungary) were used with exchangeable inserts (10 x 16 cm) coated with adhesive (Tangle Trap, Tanglefoot Co., Grand Rapids, MI, USA). Traps were placed in the foliage of *G. triacathos* "Sunburst" trees at "Lágymányosi hid", Budapest, Hungary, 2 m above ground and 10 m apart. In the first experiment, catches in traps baited with racemic (*Z*)-2-acetoxy-8-heptadecene (20 μg) and the *(R)*- or *(S)*-enantiomers (10 μg) were compared in a randomized complete block design with 7 replicates from 1–3 June 2007. In the second experiment, catches with the *(R)*- or *(S)*-enantiomers of (*Z*)-2-acetoxy-8-heptadecene and a 1:1 mixture were compared at various doses with three replicates from 14–17 June 2008. Mean catches were transformed to log(x+1) in order to normalize variances before carrying out analysis of variance (ANOVA). If the *F* value was significant for treatments ($P < 0.05$), differences between means were tested for significance by the Games-Howell *post-hoc* test, using the program SuperANOVA® (Abacus Concepts Inc., Berkeley, CA, USA).

Results

Mating Behavior Freshly-hatched *D. gleditchiae* females were observed to keep their ovipositors extended while sitting on fresh leaflets waiting for males. Otherwise, no specific calling posture was observed. Females were ready to copulate immediately after emergence. Copulations took place most frequently in the morning hours between 0800 – 1200 h under the experimental conditions. Copulations lasted on average for 19.64 sec ± 1.74 sec S.E. ($N = 25$). After mating, the female withdrew her ovipositor.

Electrophysiological Analysis of Collected Volatiles The EAG response evoked from the antenna of a male *D. gleditchiae* by stimulation with a 200 FEH aliquot of the collection of volatiles from virgin females was 0.250 mV ± 0.016 mV S.E., which was significantly stronger than that to the hexane stimulus 0.043 mV ± 0.004 mV ($P < 0.05$).

GC-FID/EAD analysis of a 150 FEH aliquot of the volatile collection from females revealed a single EAG

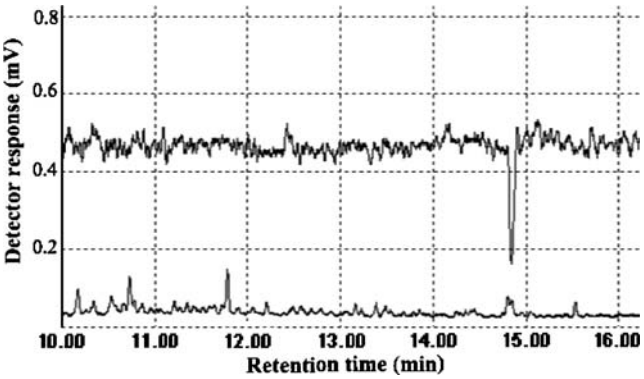

Fig. 2 Analysis by gas chromatography with flame ionization detection and simultaneous electroantennographic detection (GC-FID/EAD) of a collection of volatiles from unmated *Dasineura gleditchiae* females collected in a closed-loop stripping apparatus (CLSA). (150 female equivalent hours (FEH) injected)

response from the male antenna at 14.84 min corresponding to a noticeable peak in the FID trace (Fig. 2), and this was assumed to be a component of the female sex pheromone. Retention times of synthetic standards in parallel runs were: decyl acetate 8.80 min; tetradecyl acetate 12.99 min; hexadecyl acetate 14.87 min. Thus, the retention index of the EAG-active peak relative to saturated acetates was 1597 (Table 1).

Analyses by GC-MS GC-MS analyses of volatiles collected from female *D. gleditchiae* on the polar GC column showed a peak at retention index 1590 relative to straight chain acetates (Table 1). The mass spectrum (Fig. 3) showed ions at *m/z* 43 and 61, characteristic of acetate esters, and the highest ion at *m/z* 236. A compound having the same mass spectrum was observed at retention index 1588 relative to acetate esters in analyses on the non-polar GC column (Table 1). The similarity in retention indices relative to acetate esters on the two columns indicated the compound was probably a mono-acetate rather than di-acetate. The ion at *m/z* 236 would thus correspond to a 17–carbon acetate with one double bond after loss of acetic acid.

A small peak (4% relative to the above component) was observed in GC-MS analyses of volatile collections having mass spectrum and retention times on both GC columns identical to those of 2-acetoxyheptadecane (Table 1), and no traces (< 0.2%) of other acetoxyheptadecanes were detected. It was considered that the acetoxy group of the main pheromone component was probably in the 2-position, and this was confirmed by catalytic hydrogenation of a collection of volatiles which gave 2-acetoxyheptadecane only.

Reaction of an aliquot from the volatile collections with DMDS and subsequent analysis by GC-MS gave a derivative with strong ions at *m/z* 390 (M^+), 173, and 157 (Table 1 and Fig. 3). The latter ions correspond to $(C_8H_{17}\text{-}CHSCH_3)^+$ and $(C_7H_{13}\text{-}CHSCH_3)^+$, respectively, indicating that the double bond was in the 8-position.

(*Z*)-2-Acetoxy-8-heptadecene (Z8-17:2Ac) was synthesized via addition of methylmagnesium iodide to (*Z*)-7-hexadecenal, and had identical retention times (Table 1) and mass spectrum (Fig. 3) to those of the natural EAG-active compound. The (*E*)-2-acetoxy-8-heptadecene, present as a minor impurity of the *Z* isomer, and (*Z*)-2-acetoxy-12-heptadecene eluted later on both GC columns (Table 1).

As mentioned above, 2-acetoxy-heptadecane was present in collections of volatiles from female *D. gleditchiae* at approx 4% of the (*Z*)-2-acetoxy-8-heptadecene. (*Z*)-8-Heptadecen-2-ol could not be detected (< 0.5%). The best collection of volatiles made from 1,800 female *D. gleditchiae* contained approximately 200 ng of pheromone, corresponding to approximately 0.11 ng per female.

Absolute Configuration of Pheromone The enantiomers of (Z)-2-acetoxy-8-heptadecene were resolved easily on a cyclodextrin GC column (Fig. 4; retention times *(S)*- 19.74 min, *(R)*- 20.15 min). The individual enantiomers were prepared in high enantiomeric excess by kinetic resolution with the lipase from *Candida antarctica*, which selectively hydrolyses or acetylates the *(R)*-enantiomer (e.g., Xiao and Kitazume 1997; Gries et al. 2006). Analysis of a collection

Table 1 GC Retention indices for natural and synthetic compounds

Compound	Polar GC column[a]		Non-polar GC column[b]	
	RI[c]	KI[d]	RI[c]	KI[d]
EAD-Active compound in GC-EAD	1597			
EAD-Active compound in GC-MS	1590	2292	1588	1996
(*Z*)-2-Acetoxy-8-heptadecene	1590	2292	1588	1996
(*E*)-2-Acetoxy-8-heptadecene	1594	2296	1595	2002
(*Z*)-2-Acetoxy-12-heptadecene	1610	2311	1603	2011
(*Z*)-8-Heptadecen-2-ol	1649	2351	1480	1888
2-Acetoxyheptadecane	1573	2272	1614	2024
DMDS derivative of active compound			2226	2634

[a] DB-Wax for GC-EAD; Supelcowax 10 for GC-MS; [b] VF5; [c] GC retention time relative to retention times of acetate esters of primary, straight-chain alcohols; [d] GC retention times relative to retention times of *n*-alkanes

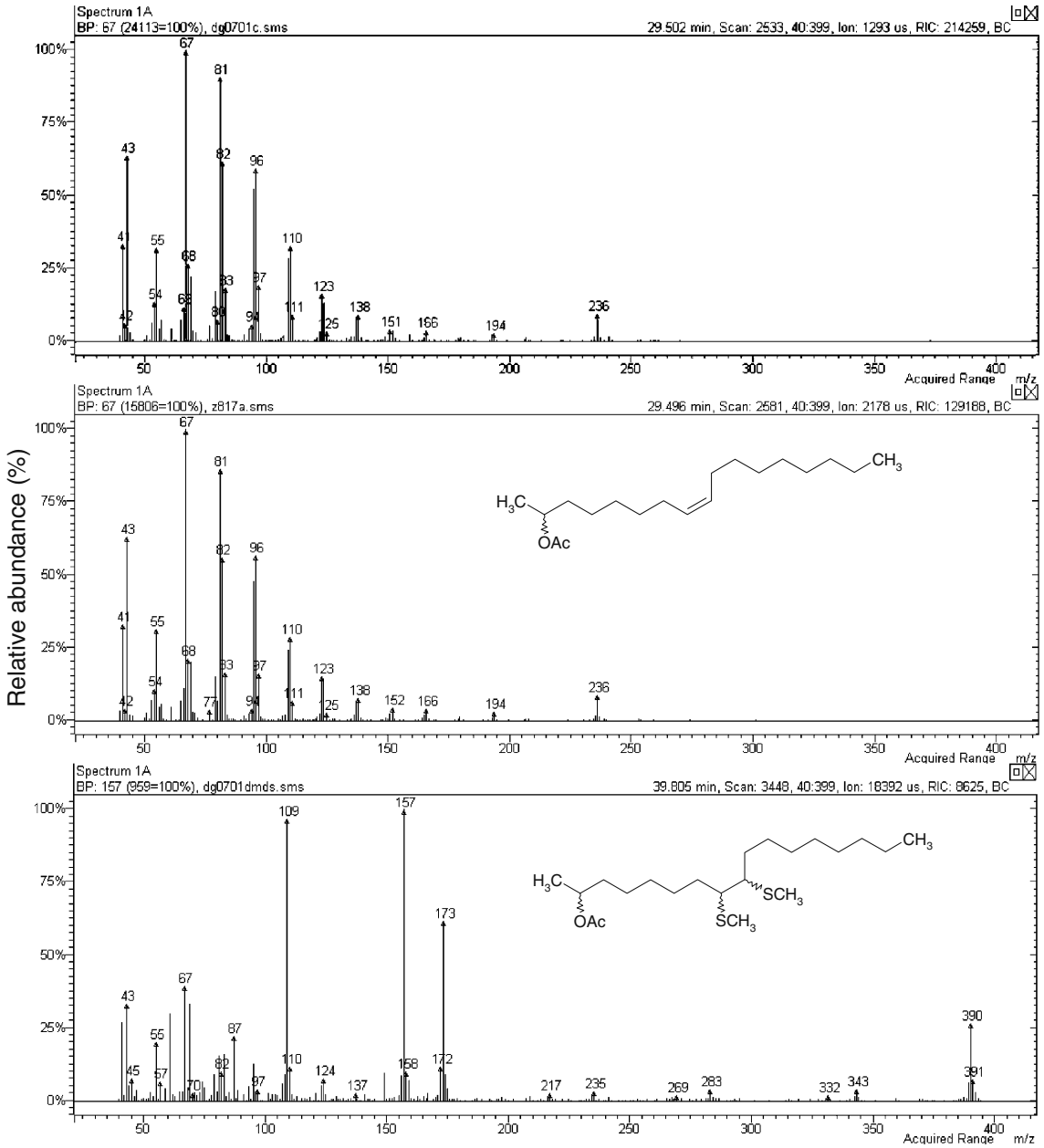

Fig. 3 Mass spectra of EAG-active compound in volatiles collected from virgin female *Dasineura gleditchiae* (upper), synthetic (*Z*)-2-acetoxy-8-heptadecene (middle) and DMDS derivative of EAG-active compound (lower)

of volatiles from female *D. gleditchiae* showed a peak at the retention time of the *(R)*-enantiomer and essentially none (< 1%) of the *(S)*-enantiomer. This was confirmed by co-chromatography of the natural compound and synthetic *(R)*-enantiomer (Fig. 4).

Electroantennography of Synthetic Compounds Both racemic (*Z*)-2-acetoxy-8-heptadecene and the *(R)*-enantiomer evoked stronger antennal responses ($P < 0.05$) from male *D. gleditchiae* than the hexane solvent across the dose range of 1 – 10 ng (Fig. 5). The *(S)*-enantiomer elicited a stronger response than hexane ($P < 0.05$) only at the 3 ng dose. The racemic sample evoked stronger responses ($P < 0.05$) than the *(S)*-enantiomer at 3 and 10 ng doses. The response evoked by an aliquot of the hexane solution of the collection of volatiles from virgin female *D. gleditchiae* containing ca. 2.8 ng of the EAG-active component fits well into the range of responses evoked by the *(R)*-enantiomer at the corresponding dose range.

Field Trapping Tests In experiment 1, traps baited with racemic (*Z*)-2-acetoxy-8-heptadecene or with the *(R)*- or *(S)*-enantiomer attracted different numbers of male *D. gleditchiae* midges ($F_{3,24} = 101.2$, $P = 0.001$). The *(R)*-enantiomer was

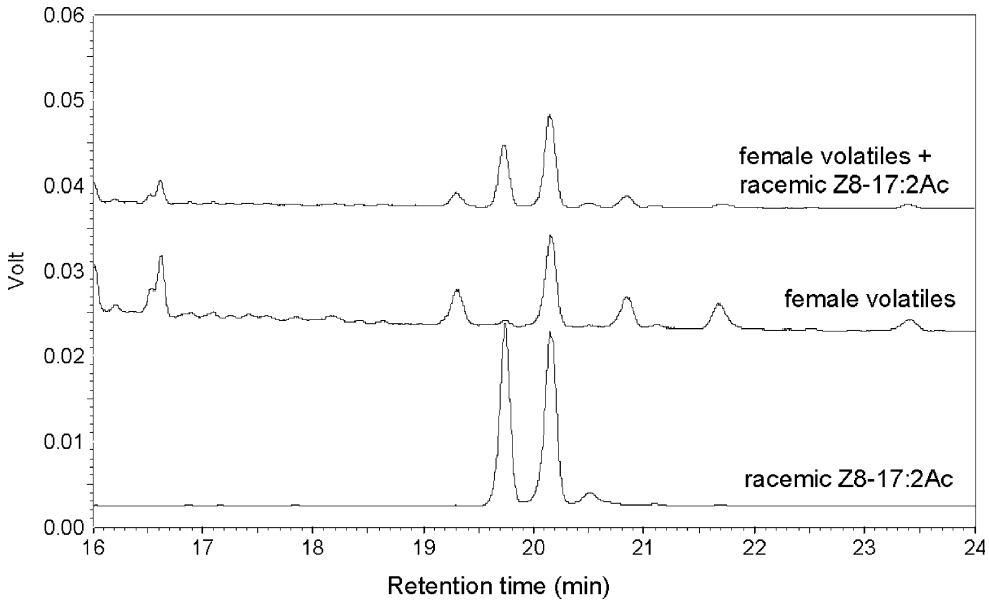

Fig. 4 GC analyses of EAG-active compound in volatiles collected from virgin female *Dasineura gleditchiae* and (*Z*)-2-acetoxy-8-heptadecene (Z8-17:2Ac) on an enantioselective cyclodextrin column

more attractive than the racemate ($P < 0.05$) (Table 2). Traps baited with the *(S)*-enantiomer caught 34 times fewer male midges than those baited with the racemate, although this was still significantly more than in unbaited control traps ($P < 0.05$), possibly due to the small amount of *(R)*-enantiomer present (Table 2).

In experiment 2, although the ANOVA indicated significant differences between treatments ($F_{8,18} = 6.436$; $P < 0.001$), traps baited with the *(R)*-enantiomer of (*Z*)-2-acetoxy-8-heptadecene at 1, 3, 10, or 30 μg or the racemate at 2, 6, 20, or 60 μg captured similar numbers of male midges ($P > 0.05$) (Fig. 6). For all these treatments, except the racemate at 2 μg or 6 μg, catches of midges were significantly greater ($P < 0.05$) than those in unbaited traps.

Discussion

We observed that the precopulatory and mating behaviors of *D. gleditchiae* were rather similar to those of several

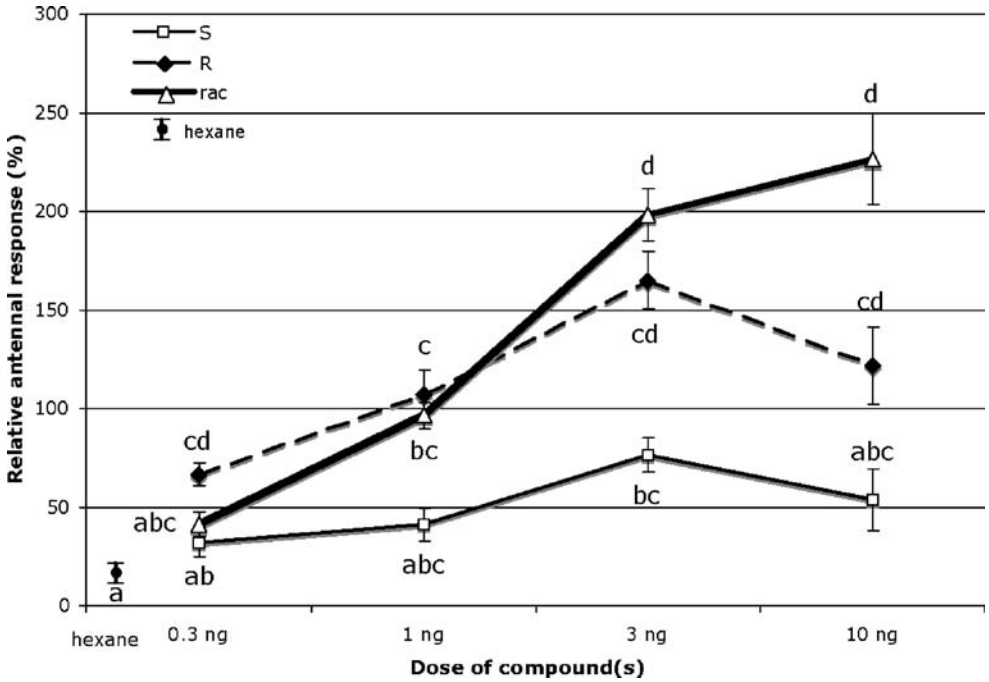

Fig. 5 EAG responses from antennae of male *Dasineura gleditchiae* to different doses of *(S)*- and *(R)*-enantiomers and racemic (*Z*)-2-acetoxy-8-heptadecene (values normalized to response to 2.8 ng natural pheromone = 100%; $N = 5$; points with different letters are significantly different at the 5% probability level after ANOVA on data transformed to log(x+1) followed by the Games-Howell test)

Table 2 Mean catches of male *Dasineura gleditchiae* with enantiomers of (*Z*)-2-acetoxy-8-heptadecene in Budapest (1-3 July, 2007, 7 replicates)

Compound	Loading (μg)	Mean catch ± SE[a]
(2*R*,8*Z*)-2-Acetoxy-8-heptadecene	10	223.0 ± 44.2 a
(2*S*,8*Z*)-2-Acetoxy-8-heptadecene	10	2.4 ± 0.9 c
Racemic (*Z*)-2-acetoxy-8-heptadecene	20	81.0 ± 17.4 b
Unbaited	-	0.7 ± 0.5 d

[a] Means followed by different letters are significantly different at 5% probability level after ANOVA on data transformed to log(x+1) followed by the Games-Howell test

other cecidomyiid species with respect to the posture of females' extruded ovipositors, readiness of mating soon after emergence, diurnal rhythm of mating taking place in the sunny morning hours, and length *in copula* (Harris and Foster 1999 and references therein).

In GC-EAD analyses of collections of volatiles from virgin female *D. gleditchiae,* a single response from male antennae was consistently observed, and this corresponded to a small peak in the FID trace. This compound was assumed to be the major component of the female sex pheromone. GC-MS analyses on both polar and non-polar columns suggested this was a secondary 17-carbon acetate with one double bond, and microanalytical reactions indicated the acetoxy function was in the 2-position and the double bond in the 8-position. (*Z*)-2-Acetoxy-8-heptadecene had an identical retention time and mass spectrum compared to the natural compound. Analyses on an enantioselective GC column indicated this consisted of exclusively the *(R)*-enantiomer.

Electroantennographic dose-response measurements of the synthetic *(S)*- and *(R)*-enantiomers and racemic (*Z*)-2-acetoxy-8-heptadecene revealed that the *(R)*-enantiomer alone or mixed with the *(S)*-enantiomer evoked significant responses from the antennae of male *D. gleditchiae,* whereas the *(S)*-enantiomer did not. In field experiments, the *(R)*-enantiomer alone or the racemic mixture were attractive to males, but the *(S)*-enantiomer was not.

Taken together, these results support the conclusion that (2*R*,8*Z*)-2-acetoxy-8-heptadecene is the major component of the female sex pheromone of *D. gleditchiae*. A trace of 2-acetoxyheptadecane was found in volatiles from the females, but (*Z*)-8-heptadecen-2-ol could not be detected. This is a novel pheromone component, but is consistent with the type of structures found in other midge sex pheromone components. It has an odd number of carbon atoms and an alkanoyloxy function on the 2-carbon (Hillbur et al. 2005 and references therein). The *Z*-8 double bond seems to be particularly common, also occurring in pheromone components of the apple leaf curling midge, *Dasineura mali*, (Cross and Hall 2005; Suckling et al. 2007), the Chinese chrysanthemum gall midge, *Rhopalomyia* spp. (Liu et al. 2007), the pear leaf curling midge, *D. pyri* (Amarawardana et al. 2006), and the blackcurrant leaf midge, *D. tetensi* (unpublished). It is of interest to note that *D. gleditchiae* produces the *(R)*-enantiomer, whereas the *(S)*-enantiomer has so far been found more commonly in other midge species, and even in a moth species, *Kermania pistaciella* Ams. (Lepidoptera: Oinophilidae) (Gries et al. 2006).

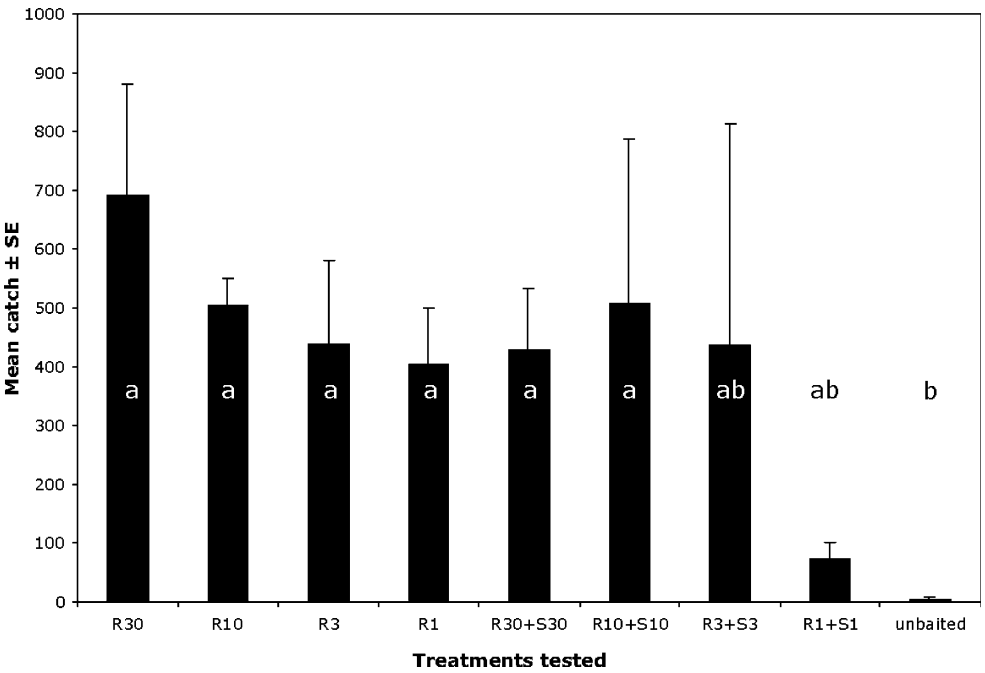

Fig. 6 Mean catches (± SE) of male *Dasineura gleditchiae* in sticky traps baited with the *(R)*-enantiomer and racemic (*Z*)-2-acetoxy-8-heptadecene at different doses at Lágymányosi hid, Budapest, Hungary. (14-17 June 2008; 3 traps/treatment. In abbreviations of treatments, the letter and number refer to the enantiomer(s) and dose, respectively, that were tested. Columns marked by the same letter do not differ significantly at the 5% probability level after ANOVA on data transformed to log(x+1) followed by the Games-Howell test)

(2*R*,8*Z*)-2-Acetoxy-8-heptadecene or the racemic material applied to red rubber septa in a dose range of 3–30 μg provides an effective bait in sticky traps for monitoring of the flight of *D. gleditchiae*. Further work is required to optimize the dose of pheromone and investigate the effects of trap positioning.

Acknowledgements This work was sponsored in part by a grant of NKFP 4/012/2004 and by a grant of the Hungarian Scientific Research Fund (OTKA) K 72767 to G. Szőcs. The GC-EAD apparatus was financed by GVOP-3.2.1-2004-04-0153/3.0 (major part) and by OTKA T37355 (minor part) to G. Szőcs. We are grateful to G. Gries for a sample of (2*S*,12*Z*)-2-acetoxy-12-heptadecene.

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(2*S*,8*Z*)-2-Butyroxy-8-heptadecene: Major Component of the Sex Pheromone of Chrysanthemum Gall Midge, *Rhopalomyia longicauda*

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Abstract The sex pheromone of the chrysanthemum gall midge, *Rhopalomyia longicauda* (Diptera: Cecidomyiidae), the most important insect pest in commercial plantations of chrysanthemum, *Dendranthema morifolium (Ramat.) Tzvel.,* in China, was identified, synthesized, and field-tested. Volatile chemicals from virgin females and males were collected on Porapak in China and sent to the United Kingdom for analysis. Coupled gas chromatographic–electroantennographic detection (GC-EAG) analysis of volatile collections from females revealed two compounds that elicited responses from antennae of males. These compounds were not present in collections from males. The major EAG-active compound was identified as 2-butyroxy-8-heptadecene by gas chromatographic (GC) retention indices, mass spectra, in both electron impact and chemical ionization modes, hydrogenation, epoxidation, and derivatization with dimethyldisulfide. The lesser EAG-active compound was identified as the corresponding alcohol. The ratio of butyrate to alcohol in the collections was 1:0.26. Racemic (*Z*)-8-heptadecen-2-ol and the corresponding butyrate ester were synthesized from (*Z*)-7-hexadecenyl acetate, and the synthetic compounds found to have identical GC retention indices and mass spectra to those of the natural, female-specific components. Analysis of the volatile collections on an enantioselective cyclodextrin GC column showed the natural pheromone contained (2*S*,8*Z*)-2-butyroxy-8-heptadecene. Field tests showed that rubber septa containing racemic (*Z*)-2-butyroxy-8-heptadecene were attractive to *R. longicauda* males. The (naturally occurring) *S*-enantiomer was equally as attractive as the racemate, while the *R*-enantiomer was not attractive to males, and did not inhibit the activity of the *S*-enantiomer. The attractiveness of the butyrate was significantly reduced by the presence of even small amounts of the corresponding alcohol.

Keywords Chrysanthemum gall midge · *Rhopalomyia longicauda* · Cecidomyiidae · Sex pheromone · (2*S*,8*Z*)-2-butyroxy-8-heptadecene · (2*S*,8*Z*)-8-heptadecen-2-ol · Field trapping

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Introduction

Chrysanthemum gall midge, *Rhopalomyia longicauda* (Diptera: Cecidomyiidae), is the most important insect pest of commercial plantations of chrysanthemum, *Dendranthema morifolium (Ramat.) Tzvel.,* in China (Cheng et al. 1990; Liu et al. 2003; Wu et al. 2007), and has also been found on wild species of chrysanthemum in Jeju Island, Korea (Sato et al. 2009). This species was first identified as

Epimgia sp. (Liu et al. 1987) in China, but was recently renamed as *Rhopalomyia longicauda* by K. M. Harris (International Institute of Entomology, UK; pers. comm.) and J. Yukawa (Entomological Laboratory, Faculty of Agriculture, Kyushu University, Japan; pers. comm.). Females lay eggs singly or in clusters near the shoot tips, the young leaves, and the flower buds. Larvae bore into and feed on the tissues of the plant, producing the conspicuous cone-shaped galls. Larval feeding causes severe distortion and stunting of the plants, resulting in a reduction in numbers and quality of flowers. Yield losses frequently exceed 40% of flowers. There are five generations of the insect per year in China, with the last generation overwintering as pupae in cocoons in the soil.

Rhopalomyia longicauda is currently controlled in China by sprays of methamidophos, oxid-dimethoate and monocrotophos as soon as galls are seen, but these insecticides are becoming less effective and are highly toxic to other organisms. It would be beneficial for control of this pest if one could predict the emergence and flight period of the adults accurately, and hence determine if and when to spray. Traps baited with sex pheromone have been developed for monitoring several other species of midge (Hillbur et al. 2000; Bruce et al. 2007; Cross et al. 2008, 2009). Here, we report identification, synthesis, and field testing of the female-produced sex pheromone of *R. longicauda*.

Methods and Materials

Experimental Insects On 30 July 2006, cone-shaped galls were picked from chrysanthemum plants in a commercial plantation in Anguo, Hebei Province, China. Pupae of *R. longicauda* were collected from these galls and transferred individually into plastic centrifuge tubes (5 ml) that contained a piece of moist filter paper. The tubes were kept at approximately 25°C and L16:D8 h. Adult midges started to emerge on 1 August. Most females emerged during 22:00–24:00 h. About 5 min after emergence, virgin females began to extend their ovipositors and apparently to release pheromone. Thus, adults were sexed and transferred to the entrainment vessel as soon as possible after emergence, so they would likely be unmated.

Collection of Volatiles Volatiles were collected from female and male *R. longicauda* placed separately in glass chambers (15×5 cm) with a glass frit at the upwind end (Hamilton Laboratory Glass, Margate, Kent, UK) at 25°C and L16:D8 h. A vacuum pump drew charcoal-filtered air (200 ml min^{-1}) through the chamber, with volatile chemicals trapped on Porapak Q (200 mg; 50/80 mesh; Waters Associates Inc., Milford, MA, USA) held between plugs of silanized glass wool in a Pasteur pipette (4 mm i.d.). The life span of adult *R. longicauda* is 1–2 d so, each day, dead midges were removed and fresh live midges introduced into the chambers. The collection filters were renewed after volatiles from approximately 300 female midges had been collected, approximately every 4 d. The collection filters were mailed to the UK where the trapped chemicals were eluted from the Porapak Q with dichloromethane (Pesticide grade; 3×0.5 ml) and concentrated to one-fifth the original volume by a gentle stream of nitrogen.

Analysis by Gas Chromatography (GC) Samples were analyzed by gas chromatography on an HP 6850 instrument (Agilent Technologies, Reading, Berks., UK) with a fused silica capillary column (30 m×0.32 mm i.d.×0.25 µm film thickness) coated with a polar phase (Wax10, Supelco, Gillingham, Dorset, UK). The oven temperature was programmed from 60°C for 2 min, then at 10°C min^{-1} to 250°C. Carrier gas was helium (2.4 ml min^{-1}), injection was splitless (220°C), and detection was by flame ionization (FID; 250°C). For all GC analyses, retention times were converted to Retention Indices (RI) relative to those for *n*-alkanes.

Analysis by Gas Chromatography-Electroantennography (GC-EAG) GC-EAG analyses were carried out with an HP 6890 instrument (Agilent) with capillary GC columns (30 m×0.32 mm i.d.×0.25 µm film thickness) coated with polar (Wax10; Supelco) and non-polar (SPB1; Supelco) phases. The oven temperature was programmed from 50°C for 2 min, then at 10°C min^{-1} to 250°C. Carrier gas was helium (2.4 ml min^{-1}), and injection was splitless (220°C). The GC column effluent was split (1:1) with a push-fit Y-piece between the FID (250°C) and a silanized glass T-piece in the column oven. Nitrogen (200 ml min^{-1}) was blown continuously over the EAG preparation, and every 17 sec this was diverted through the T-piece for 3 sec, blowing the contents over the EAG preparation, as described by Cork et al. (1990).

For the EAG preparation, the wings and legs of a male midge were excised, and the whole insect was suspended between glass electrodes containing electrolyte (0.1 M potassium chloride with 10% polyvinylpyrrolidone added to reduce evaporation). The ends of both antennae were inserted into the recording electrode and the body into the reference electrode. The electrodes were attached to silver/silver chloride electrodes held in micromanipulators on a portable EAG device developed by Syntech (INR-02; Syntech, Hilversum, The Netherlands). Both FID and EAG signals were collected and analyzed with EZChrom software (Elite v3.0; Scientific Software Inc., Pleasanton, CA, USA).

Gas Chromatography Linked to Mass Spectrometry (GC-MS) GC-MS analyses were carried out on a Carlo Erba

5130 GC (Thermoelectron, Hemel Hempstead, Hertfordshire, UK) linked directly to an ion trap detector (Finnigan ITD 700; Thermoelectron) operated in electron impact (EI), or chemical ionization (CI) with isobutane, modes. The polar GC column (30 m×0.25 mm i.d.×0.25 μm film thickness) was coated with Wax 10 (Supelco). The carrier gas was helium (0.5 kg cm^{-2}), injection was splitless (220°C), and the oven temperature was programmed from 50°C for 2 min, then at 6°C min^{-1} to 250°C.

GC-MS analyses were also carried out on an HP 6890 GC (Agilent) coupled to an HP 5973 MSD (Agilent) operated in EI mode. The GC column (30 m×0.25 mm i.d.×0.25 μm film thickness) was coated with a non-polar phase (SPB1; Supelco). The carrier gas was helium (1 ml min^{-1}), injection was splitless (220°C), and the oven temperature programmed from 60°C for 2 min, then at 6°C min^{-1} to 250°C.

Micro-analytical Reactions For hydrogenation, a sample of the concentrated volatile collection (in 20 μl dichloromethane, containing approx. 20 ng of pheromone) from female midges was placed in a conical vial (1.1 ml; Chromacol Ltd, Welwyn Garden City, UK). Most of the solvent was removed by a stream of purified nitrogen before hexane (50 μl) was added. The hydrogenation catalyst (10% palladium on charcoal; 100 mg) was placed in another vial, washed twice with hexane (1 ml), and then suspended in hexane (1 ml). An aliquot of the suspension (1 drop) was added to the hexane solution of the collected volatiles. Hydrogen was then bubbled into the mixture through a piece of fused silica capillary column for 1 min. The mixture was allowed to stand for 5 min, and then was analyzed by GC-MS using both polar and non-polar columns.

For epoxidation, a solution of 3-chloroperbenzoic acid in dichloromethane (5 μl, 1 mg ml^{-1}) was added to an aliquot of a concentrated volatile collection (in 20 μl dichloromethane, containing approx. 20 ng) from female midges. After standing at room temperature for 2 h, the reaction mixture was analyzed directly by GC-MS by using both polar and non-polar columns (Bierl-Leonhardt et al. 1980).

For reaction with dimethyldisulfide (DMDS), a sample of the concentrated collection (in 20 μl dichloromethane, containing approx. 20 ng) from female midges was transferred to a conical vial (1.1 ml). Most of the solvent was removed by a stream of purified nitrogen before hexane (20 μl) was added, followed by DMDS (20 μl; SigmaAldrich, Gillingham, Dorset, UK) and a solution of iodine in diethyl ether (2 μl; 6 mg ml^{-1}). The mixture was allowed to stand overnight at approximately 40°C. Hexane (50 μl) was added, followed by aqueous sodium thiosulfate solution (5%; 50 μl). The two layers were mixed until the iodine color had disappeared, and the lower aqueous layer was removed with a syringe. The resulting solution was dried over magnesium sulfate and analyzed by GC-MS on the non-polar column (Buser et al. 1983; Leonhardt and DeVilbiss 1985).

Enantioselective Gas Chromatography GC analyses were also carried out on a column (30 m×0.32 mm i.d.×0.25 μm film thickness) coated with a chiral cyclodextrin stationary phase (Chirasil-Dex CB; Varian, Oxford, UK). The oven temperature was programmed from 60°C for 2 min, then at 20°C min^{-1} to 165°C. Injection was splitless at 220°C with helium as the carrier gas (2.4 ml min^{-1}).

Synthesis Nuclear Magnetic Resonance (NMR) spectra were recorded in $CDCl_3$ on a JEOL EX270 machine at 270 MHz for ^{1}H and 67.8 MHz for ^{13}C. Infrared (IR) spectra were recorded as thin films with a Perkin Elmer 881 grating spectrophotometer (Perkin Elmer, Beaconsfield, Bucks., UK).

(*Z*)-2-Butyroxy-8-heptadecene was synthesized by addition of methylmagnesium iodide to (*Z*)-7-hexadecenal, followed by conversion of the resulting alcohol to the butyrate (Fig. 1). The racemic material was resolved by hydrolysis with lipase from *Candida antarctica*, which gave unreacted 2*S*-butyrate and the 2*R*-alcohol, which was separated and converted to the corresponding butyrate (Fig. 1).

(*Z*)-7-Hexadecenyl acetate, prepared previously at NRI by a standard acetylenic route, was hydrolyzed with potassium carbonate in methanol, and the resulting alcohol was oxidized to the aldehyde with pyridinium chlorochromate in dichloromethane (Corey and Suggs 1975). Methyl magnesium iodide was prepared by addition of methyl iodide (2.84 g, 20 mM) in dry diethyl ether (10 ml) to magnesium turnings (0.6 g, 25 mM) in diethyl ether (20 mM) under nitrogen. Titration indicated the resulting solution was 0.76 M. To this solution (7.5 ml, 6 mM) was added (*Z*)-7-hexadecenal (0.83 g, 3.5 mM) in diethyl ether (10 ml) dropwise with stirring and ice cooling. Standard aqueous workup and Kugelrohr distillation gave (*Z*)-8-heptadecen-2-ol (b.p. 140°C, 0.04 mm Hg; 0.7 g, 80%). IR (film): ν 3420 cm^{-1} (O-H); MS: *m/z* (%) 254 (M^{+}, 6), 236 (M-H_2O, 3), 138 (8), 137 (7), 125 (11), 124 (11), 111 (8), 110 (27), 109 (31), 97 (26), 96 (64), 95 (60), 83 (22), 82 (52), 81 (100), 69 (23), 68 (21), 67 (100), 57 (12), 55 (38), 54 (12), 45 (29), 43 (21), 41 (38).

The alcohol (0.254 g, 1 mM) was mixed with butyric acid (0.132 g, 1.5 mM) and N,N-dimethylaminopyridine (10 mg) in dichloromethane (5 ml) and N,N'-dicyclohexylcarbodiimide (0.3 g, 1.4 mM) added in portions. After stirring for 5 h at room temperature, diethyl ether (5 ml) was added, the mixture filtered, and solvents were removed on a rotary evaporator. The residue was chromatographed

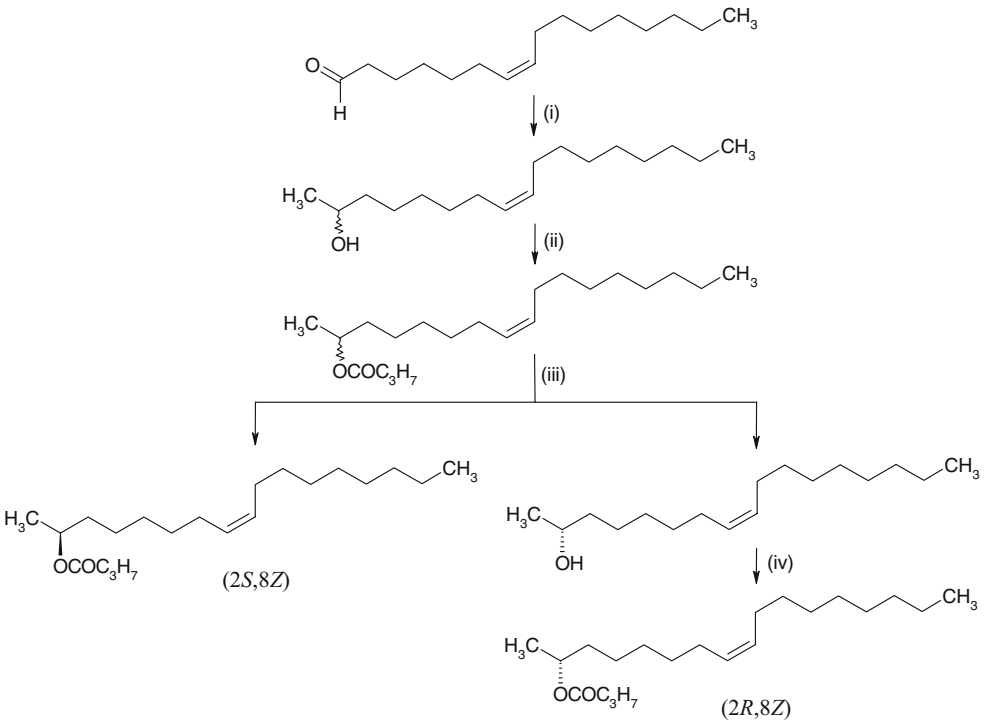

Fig. 1 Synthesis and resolution of enantiomers of (*Z*)-2-butyroxy-8-heptadecene. Reagents: (i) MeMgI in diethyl ether; (ii) butyric acid/ N,N-4-dimethylaminopyridine/ N,N'-dicyclohexylcarbodiimide in dichloromethane; (iii) lipase from *Candida antarctica* in phosphate buffer pH 7; (iv) butyryl chloride/pyridine in dichloromethane)

on silica gel (12 g) using 2% diethyl ether in petroleum ether, and the product was distilled in a Kugelrohr apparatus to give (*Z*)-2-butyroxy-8-heptadecene containing approximately 5% of the *E* isomer (b.p. 180°C/ 0.04 mm Hg; 0.25 g, 78%). IR (film): ν 1735 cm^{-1} (C = O); ^{1}HNMR: δ 0.88 (t, *J*=6.8 Hz, 3H, CH_3-CH_2), 0.95 (t, *J*= 7.3 Hz, 3H, CH_3-CH_2-CH_2-CO), 1.20 (d, *J*=6.6 Hz, 3H, CH_3-CH), 1.2–1.4 (br m, 18H, -CH_2-), 1.4–1.4 (br m, 2H), 1.65 (hex, *J*=7.6, 2H, CH_3-CH_2-CH_2-CO), 1.95–2.05 (br m, 4H, CH_2-CH=), 2.25 (t, 2H, *J*=7.6, CH_3-CH_2-CH_2-CO), 4.90 (hex, J=6.2 Hz, 1H, CH_3-CH-O), 5.3–5.4 (m, 2H, C = CH); ^{13}C NMR: δ 13.68, 14.12, 18.60, 20.05, 22,69, 25.33, 27.11, 27.24, 29.11, 29.34, 29.54, 29.64, 29.78, 31.92, 35.95, 36.67, 70.72, 129.65, 130.09, 173.38.

(*Z*)-2-Butyroxy-8-heptadecene (0.25 g, 0.8 mM) was stirred with dipotassium hydrogenphosphate buffer (0.1 M, pH 7; 3 ml) and lipase acrylic resin from *Candida antarctica* (Sigma-Aldrich; 25 mg) overnight at room temperature. The mixture was extracted twice with 10% diethyl ether in petroleum ether (10 ml), and the extracts were washed once with brine (10 ml) and dried over magnesium sulfate. After removal of solvent on a rotary evaporator, the residue (0.25 gm) was chromatographed on silica gel (10 g), and eluted with a gradient of diethyl ether in petroleum ether from 2% to 20%. The unreacted ester fraction contained (2*S*,8*Z*)-2-butyroxy-8-heptadecene (0.11 g) with an enantiomeric excess (ee) of 99.8% by GC analysis on the enantioselective column. The alcohol fraction (0.11 g) was dissolved in dichloromethane, and pyridine (50 mg) and butyryl chloride (100 mg) were added. After stirring at room temperature overnight, standard aqueous workup gave (2*R*,8*Z*)-2-butyroxy-8-heptadecene (0.12 g) with 99.1% ee. (2*S*,8*Z*)-8-Heptadecen-2-ol for use in field tests was prepared by hydrolysis of (2*S*,8*Z*)-2-butyroxy-8-heptadecene (20 mg) with potassium hydroxide in ethanol (0.1 N, 1 ml) for 8 h.

2-Butyroxyheptadecane was prepared from 2-heptadecanone synthesized previously at NRI by addition of methyl lithium to hexadecanoic acid in ether (Jorgenson 1970). The ketone was reduced to 2-heptadecanol with sodium borohydride in aqueous ethanol, and reacted with butyric acid, N,N-dimethylaminopyridine and N,N'-dicyclohexylcarbodiimide in dichloromethane (b.p. 160°C/ 0.03 mm Hg). IR (film): ν 1735 cm^{-1}; MS: *m/z* (%) 238 (5), 139 (3), 125 (7), 115 (21), 111 (14), 97 (23), 89 (33), 85 (12), 83 (20), 71 (100), 69 (19), 67 (5), 57 (44), 55 (38), 43 (77), 41 (34).

(2*S*,12*Z*)-2-Acetoxy-12-heptadecene, the pheromone of the pistachio twig borer, *Kermania pistaciella* (Gries et al. 2006), was kindly provided by Professor Gerhard Gries. A sample was hydrolyzed with potassium carbonate in methanol to give (2*S*,12*Z*)-8-heptadecen-2-ol, and this was reacted with butyryl chloride and pyridine in dichloromethane to give a sample of (2*S*,12*Z*)-2-butyroxy-12-heptadecene.

Field Experiments Field experiments were conducted in commercial chrysanthemum plantations in Angou, Hebei Province, China. Dispensers were red rubber septa (20× 10 mm o.d., International Pheromone Systems Ltd., Wirral,

UK) impregnated with the test chemical(s) applied in petroleum ether (0.1 ml) containing 2,6-di-*tert*-butyl-4-methylphenol (BHT, 99%; Sigma-Aldrich) as an antioxidant, at 20% the amount of the major test component. Traps were sticky delta traps (20 cm long×17 cm sides) with the septum fixed 2 cm above the sticky insert. Experiments employed a randomized complete block design with 3 replicates each, and traps were placed at 20-m intervals.

Experiment 1 was carried out from 30 June to 3 July 2007, when plants were 25–35 cm high. Traps were hung 30 cm above the ground. Treatments were (A) racemic (*Z*)-2-butyroxy-8-heptadecene (20 µg); (B) (*Z*)-2-butyroxy-8-heptadecene (20 µg)+(*Z*)-8-heptadecen-2-ol (3 µg); (C) (2*S*,8*Z*)-2-butyroxy-8-heptadecene (10 µg); (D) (2*S*,8*Z*)-2-butyroxy-8-heptadecene (10 µg) + (2*S*,8*Z*)-8-heptadecen-2-ol (1.5 µg); (E) (2*R*,8*Z*)-2-butyroxy-8-heptadecene (10 µg); (F) (2*R*,8*Z*)-2-butyroxy-8-heptadecene (10 µg) + (2*R*,8*Z*)-8-heptadecen-2-ol (1.5 µg); and (G) unbaited control.

Experiment 2 was carried out from 19 to 23 September 2007 when plants were 70–80 cm high. Traps were hung 75 cm above the ground. There were 7 treatments: (2*S*,8*Z*)-2-butyroxy-8-heptadecene (10 µg), five two-component blends of (2*S*,8*Z*)-2-butyroxy-8-heptadecene (10 µg) with (2*S*,8*Z*) -8-heptadecen-2-ol at 1, 2, 5, 10, and 15% of the major component, and unbaited control.

Catches of male *R. longicauda* were transformed to log(x+1) to improve the equality of variances, as judged by plots of residuals against estimated means, and subjected to two-way analysis of variance (ANOVA), excluding treatments in which no midges were caught (Genstat for Windows version 10, VSN International Ltd., Hemel Hempstead, UK). Differences between means were tested for significance at the 5% level by the Least Squares Difference (LSD) test.

Release Rate Measurements For release-rate measurements, two rubber septa dispensers were loaded with a 1:1 blend of racemic (*Z*)-2-butyroxy-8-heptadecene and (*Z*)-8-heptadecen-2-ol (50 µg each), and maintained in a laboratory windtunnel at 27°C and 8 km/h windspeed. At intervals, volatiles were collected from each septum separately as described for collection of volatiles from live insects, except that the septa were held in smaller glass vessels (10×3 cm) constructed from Quickfit adapters. Volatiles were collected on Porapak for 24 h at a flow rate of 2 l min^{-1}. Collected volatiles were eluted with dichloromethane, pentadecyl acetate (200 ng) was added as an internal standard, and the solution was analyzed by GC-MS on the polar column.

Results

Pheromone Identification GC-FID analyses of three collections from female *R. longicauda* midges and three collections from male midges, using a polar GC column, showed two compounds present in all the collections from females; neither of these components were present in collections from males. GC-EAG analyses of volatiles from virgin female *R. longicauda* on both polar and non-polar GC columns revealed two compounds that elicited responses from the antennae of males (Fig. 2); again, these compounds were not detectable in collections from virgin males. RIs for the EAG-active compounds are shown in Table 1.

Collections of volatiles from female midges were analyzed by GC-MS using both polar and non-polar columns. The EI fragmentation pattern of the major EAG-active compound (Fig. 3) suggested that it was a butyrate, as evidenced by ions at *m/z* 71 ($C_3H_7CO^+$) and *m/z* 89 ($C_3H_7COOH_2^+$). The ion at *m/z* 236 corresponded to loss of butyric acid from a 17-carbon butyrate with one double bond (molecular weight 324). The CI mass spectrum showed ions at *m/z* 326 and 237 confirming this.

After catalytic hydrogenation of a sample of volatiles from female *R. longicauda*, GC-MS analysis showed the major compound had disappeared and was replaced by a butyrate ester having GC RIs and mass spectrum identical with those of 2-butyroxyheptadecane (Table 1). The EI mass spectrum of this compound showed ions at *m/z* 238 and at *m/z* 115 (CH_3-CH-$OOCC_3H_7^+$), confirming that the butyrate was in the C-2 position.

Epoxidation of a sample from female *R. longicauda* gave a product (RI 2363 on the non-polar GC column, 2822 on the polar column) with an EI mass spectrum that showed an ion at *m/z* 252, corresponding to loss of butyric acid from the epoxide of a monounsaturated 17-carbon butyrate. Ions at *m/z* 155 (C_8H_{17}-$C_2H_2O^+$) and *m/z* 109 [loss of butyric acid from CH_3-CH($OOCC_3H_7$)-$(CH_2)_5$-$C_2H_2O^+$] indicated that the double bond in the original compound was in the C-8 position. This was confirmed by reaction of a sample from female *R. longicauda* with DMDS, which gave a product (RI 2809 on the non-polar column) with a mass spectrum that showed a molecular ion at *m/z* 418, corresponding to addition of one DMDS molecule (94) to a compound with molecular weight of 324. Diagnostic ions at *m/z* 173 [$CH_3(CH_2)_8$-$CHSCH_3^+$] and *m/z* 137 [loss of butyric acid from CH_3-CH($OOCC_3H_7$)-$(CH_2)_5$-$CHSCH_3^+$] confirmed that the double bond was located at the C-8 position. (*Z*)-2-Butyroxy-8-heptadecene was synthesized and found to have identical RIs and mass spectra to those of the major EAG-active compound in volatiles from female *R. longicauda*. The RIs were different from those of the corresponding *E*-isomer, present as an impurity in the *Z*-isomer, and those of (*Z*)-2-butyroxy-12-heptadecene, derived from (*Z*)-2-acetoxy-12-heptadecene, the pheromone of the pistachio twig borer, *Kermania pistaciella* (Gries et al. 2006) (Table 1).

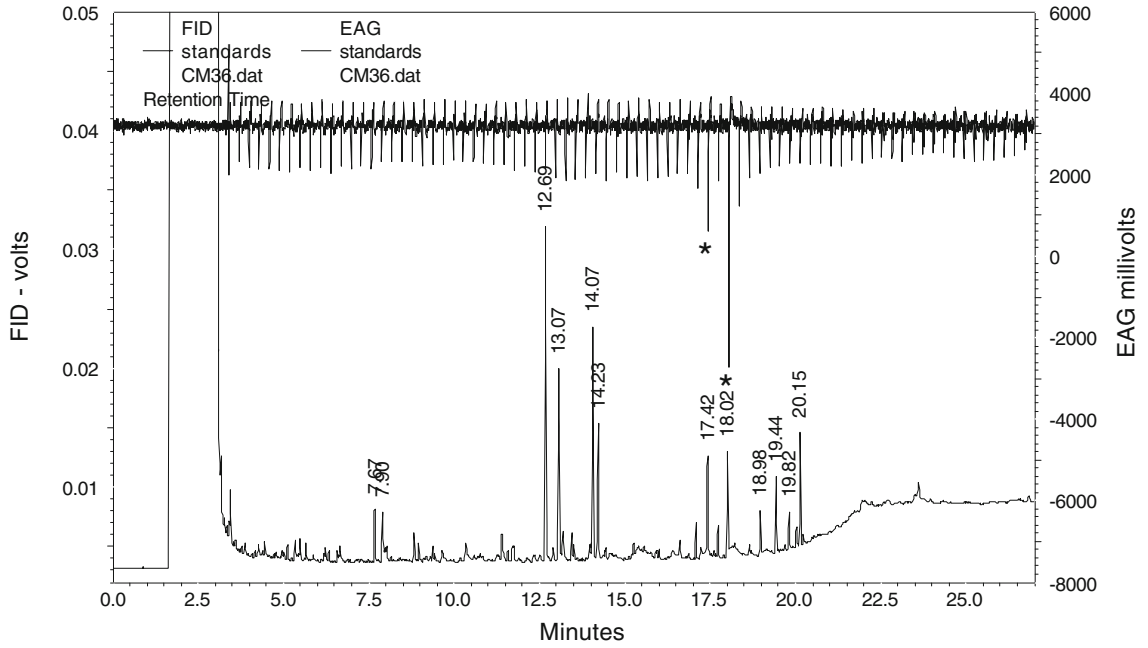

Fig. 2 Coupled gas chromatographic–electroantennographic (GC-EAG) detection analysis of volatiles collected from virgin female *Rhopalomyia longicauda* using a male electroantennogram preparation and a polar column (EAG responses are marked with *)

The mass spectrum of the minor EAG-active compound showed a molecular ion at *m/z* 254 and an ion at *m/z* 237 (M-17), indicating a monounsaturated 17-carbon alcohol. An ion at *m/z* 45 [CH_3-$CH(OH)^+$] was consistent with the alcohol group being at the C-2 position. Synthetic (*Z*)-8-heptadecen-2-ol was found to have identical GC RIs (Table 1) and mass spectrum to those of this compound (Table 1).

The proportion of the minor, relative to the major, compound in the volatiles from female *R. longicauda* was 0.26:1±0.06 (SEM; *N*=3), determined by integration of the total ion trace in GC-MS analyses on the polar column. The minor compound co-chromatographed with an impurity on the column used for GC-EAG analyses in Fig. 2. Comparison of peak areas with those of standard acetate esters

Table 1 Gas chromatographic retention indices (RI), relative to *n*-alkanes, for the major and minor electroantennogram-active components in the volatile collections of female *Rhopalomyia longicauda* and various synthetic compounds, on polar (Wax10) and non-polar (SPB1) columns

	RI	
Compound	Wax10	SPB1
Major electroantennogram-active component	2418	2171
Minor electroantennogram-active component	2348	1885
(*Z*)-2-butyroxy-8-heptadecene (I)	2418	2172
(*E*)-2-butyroxy-8-heptadecene	2423	2178
(*Z*)-2-butyroxy-12-heptadecene	2442	2188
(*Z*)-8-heptadecen-2-ol (II)	2353	1885
(*Z*)-12-heptadecen-2-ol	2311	1907
2-heptadecyl butyrate	2399	2197
2-heptadecanol	2331	1909

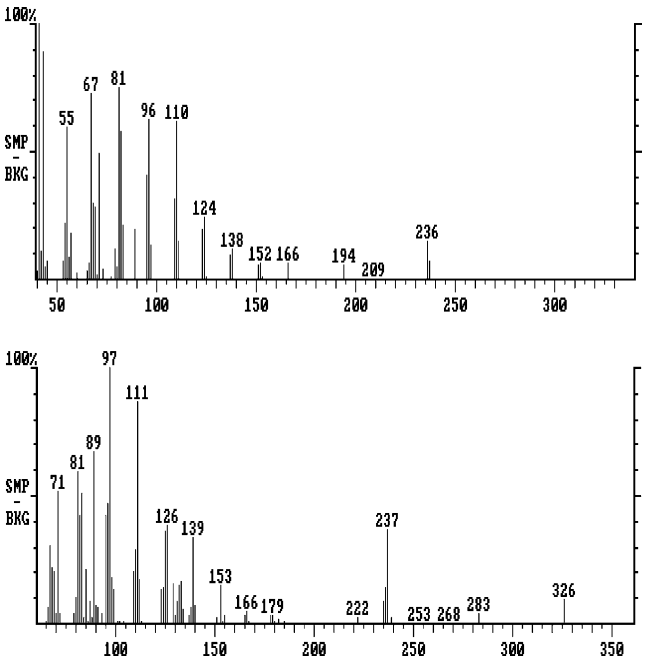

Fig. 3 Mass spectra of the major pheromone component of *Rhopalomyia longicauda* using electron impact (upper) and chemical ionization (lower)

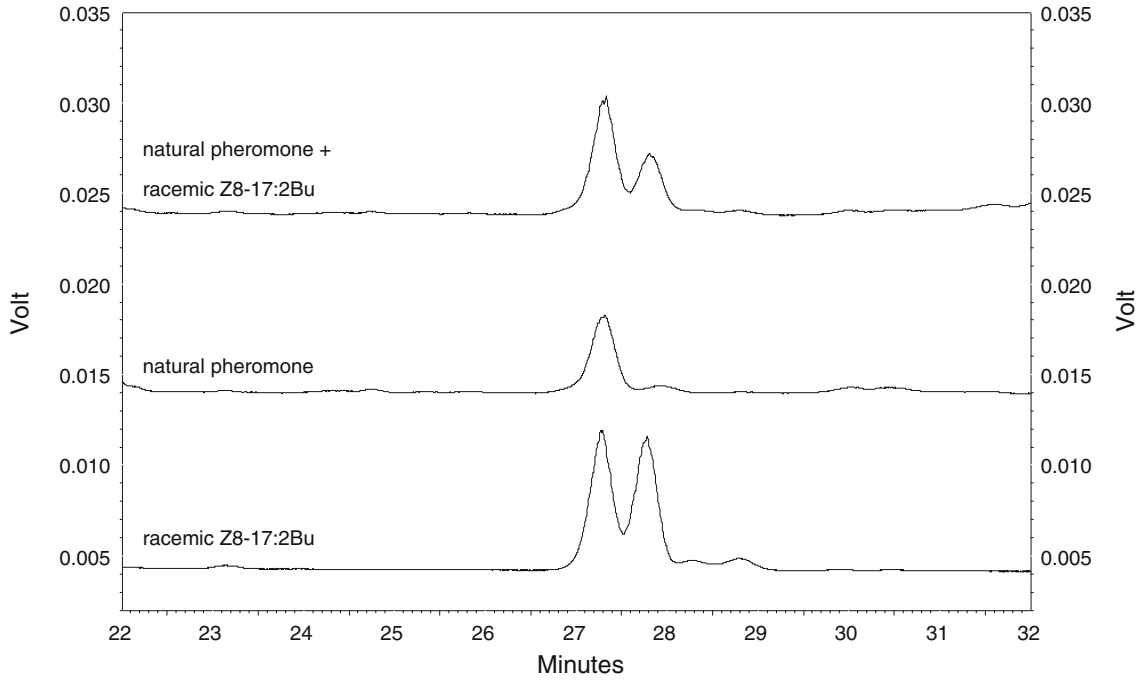

Fig. 4 Gas chromatography (GC) analyses of natural and synthetic pheromone components on an enantioselective GC column: racemic (*Z*)-2-butyroxy-8-heptadecene (Z8-17:2Bu) (lower); volatiles from female *Rhopalomyia longicauda* (middle); coinjection of racemic (*Z*)-2-butyroxy-8-heptadecene and volatiles from female *R. longicauda* (upper)

indicated that the amount of the major compound produced was up to 0.7 ng/female over a life of 1–2 days.

Configuration of the Two Components The two enantiomers of synthetic (*Z*)-2-butyroxy-8-heptadecene were separated on a cyclodextrin column, with the *S* enantiomer eluting at 27.25 min and the *R* enantiomer at 27.75 min (Fig. 4). The identities of the two enantiomers were resolved following enantioselective hydrolysis with lipase from *Candida antarctica*, which selectively hydrolyses the *R*-enantiomer (e.g., Xiao and Kitazume 1997; Gries et al. 2006). Analysis of volatiles from female *R. longicauda* showed a peak at the same retention time as the first (i.e., the S-enantiomer) of the peaks in the racemate, and no peak corresponding to the second-eluting (i.e., R-) enantiomer (Fig. 4). The natural chemical co-chromatographed with the first-eluting enantiomer, confirming its identity as (2*S*,8*Z*)-2-butyroxy-8-heptadecene (Fig. 4). The two enantiomers of (*Z*)-8-heptadecen-2-ol could not be separated on this column.

Field Tests Field-trapping tests were conducted to determine the activity of synthetic (*Z*)-2-butyroxy-8-heptadecene, (*Z*)-8-heptadecen-2-ol, and their blends. In Experiment 1, differences among treatments were highly significant (Fig. 5; $F=11.78$, df 5,10, $P<0.001$). Traps baited with synthetic racemic (*Z*)-2-butyroxy-8-heptadecene (A) or (2*S*,8*Z*)-2-butyroxy-8-heptadecene (C) caught equal numbers of male *R. longicauda* ($P>0.05$), while traps baited with (2*R*,8*Z*)-2-butyroxy-8-heptadecene (E) caught no males. Addition of the alcohol to the butyrate at a ratio of 0.15:1, corresponding to the ratio found in volatiles from female *R. longicauda* midges, completely suppressed trap

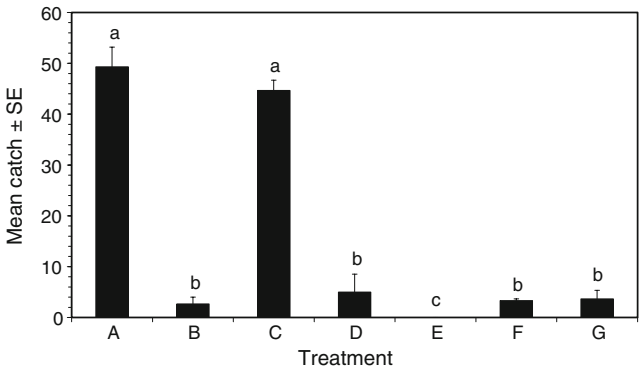

Fig. 5 Mean catchers (±SE) of male *Rhopalomyia longicauda* captured in traps baited with synthetic candidate pheromone components: (A) racemic (*Z*)-2-butyroxy-8-heptadecene (20 μg); (B) (*Z*)-2-butyroxy-8-heptadecene (20 μg) + (*Z*)-8-heptadecen-2-ol (3 μg); (C) (2*S*,8*Z*)-2-butyroxy-8-heptadecene (10 μg); (D) (2*S*,8*Z*)-2-butyroxy-8-heptadecene (10 μg) + (2*S*,8*Z*)-8-heptadecen-2-ol (1.5 μg); (E) (2*R*,8*Z*)-2-butyroxy-8-heptadecene (10 μg); (F) (2*R*,8*Z*)-2-butyroxy-8-heptadecene (10 μg) + (2*R*,8*Z*)-8-heptadecen-2-ol (1.5 μg); and (G) unbaited control. Trial carried out from 30 June – 3 July 2007; 3 replicates; totals with different letters are significantly different, $P<0.05$

capture of male midges. Thus, traps baited with racemates of both compounds (B), with the *S*-enantiomers of both compounds (D), or with the *R*-enantiomers of both compounds (F) caught numbers of males that were not significantly different from those captured in unbaited traps (G) ($P>0.05$) (Fig. 5).

Determination of release rates of (*Z*)-2-butyroxy-8-heptadecene and (*Z*)-8-heptadecen-2-ol from a rubber septum loaded with equal amounts of the two compounds (50 μg each) showed that the mean ratio of the alcohol to the butyrate in the volatiles released was 13.3:1 after 2 d, 13.3:1 after 4 d, 12.1:1 after 9 d and 10.4:1 after 15 d. Thus, a blend containing 0.02:1 alcohol:butyrate on a septum would be expected to give a release ratio of components similar to the 0.26:1 blend released by female *R. longicauda*. Experiment 2, therefore, examined the effect on trap catches of adding (2*S*,8*Z*)-8-heptadecen-2-ol to (2*S*,8*Z*)-2-butyroxy-8-heptadecene at five different ratios from 0.01:1 to 0.15:1. All blends were significantly less attractive ($P<0.05$) than (2*S*,8*Z*)-2-butyroxy-8-heptadecene alone; the 0.1:1 and 0.15:1 blends caught no male midges at all (Fig. 6; $F=43.62$, *df* 3,6, $P<0.001$).

Discussion

(2*S*,8*Z*)-2-Butyroxy-8-heptadecene is released by virgin female *R. longicauda* midges and not by males. This compound elicited an EAG response from conspecific males and was highly attractive to male midges in field trapping tests, although no direct comparison with the attractiveness of live females or extracts of the pheromone glands of females was performed. Thus, this compound is the major component of the sex pheromone produced by female *R. longicauda*. The *R*-enantiomer of this compound was unattractive to males, and did not appear to affect the attractiveness of the *S*-enantiomer when the racemate was tested.

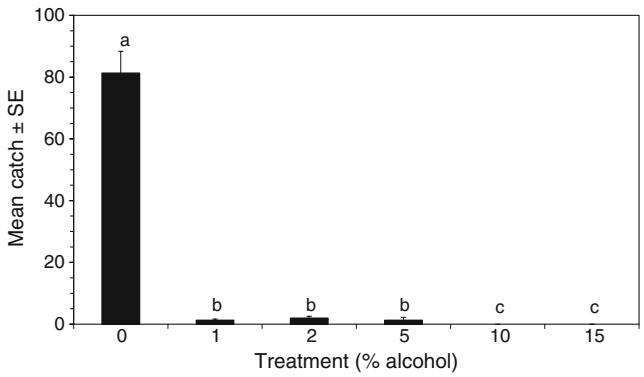

Fig. 6 Mean catches (±SE) of male *Rhopalomyia longicauda* captured in traps baited with blends containing different ratios of candidate pheromone components (2*S*,8*Z*)-2-butyroxy-8-heptadecene (10 μg) and (2*S*,8*Z*)-8-heptadecen-2-ol. Trial carried out from 19–23 September 2007; 3 replicates; totals with different letters are significantly different, $P<0.05$

The corresponding alcohol, (*Z*)-8-heptadecen-2-ol also was present in volatile collections from female *R. longicauda* midges in a ratio of approximately 0.26:1, relative to the butyrate, and this compound also elicited an EAG response from antennae of male midges. Determination of relative release rates of the butyrate and alcohol from the rubber septa used as dispensers in field tests indicated that a ratio of 0.02:1 alcohol: butyrate on the septum should release a blend approximating that found in volatile collections from female midges. However, even at ratios as low as 0.01:1, the presence of the alcohol suppressed the attractiveness of the butyrate to males in field trials.

Since the alcohol appears to inhibit attraction to the butyrate, its presence in the volatile collections is surprising. It is unlikely that the alcohol arose from the butyrate by decomposition during the collection process. Re-examination of traces from GC-FID analyses of samples immediately after extraction from the collection filters showed the presence of the alcohol in similar relative proportions to those observed during GC-EAG analyses carried out on samples stored for three months. The enantiomers of the alcohol could not be separated on the enantioselective GC column used, and it was assumed that the female midges produced the *S*-enantiomer exclusively as they do for the butyrate. Hence, we tested only the S-enantiomer or the racemic mixture, which both suppressed trap capture when added to the butyrate. Another possibility is that the *R*-enantiomer of the alcohol is attractive and the *S*-enantiomer is strongly inhibitory. This should be investigated in future studies.

Neither (2*S*,8*Z*)-2-butyroxy-8-heptadecene nor the corresponding alcohol have been reported as insect pheromone components. However, the structure of these compounds conforms to the general pattern of structures found in pheromone components of other cecidomyiid midges. The backbone has an odd number of carbon atoms with an oxygenated functionality in the 2-position (Hall et al. 2009). The opposite enantiomer of the corresponding acetate, (2*R*,8*Z*)-2-acetoxy-8-heptadecene, is the pheromone of the honey locust gall midge, *Dasineura gleditchiae* (Molnár et al. 2009), and (*Z*)-13-acetoxy-8-heptadecen-2-one is the pheromone of the apple leaf curling midge, *D. mali* (Cross and Hall 2005; Suckling et al. 2007; Cross et al. 2009). The butyrate ester functionality has also been found in the sex pheromone component, (2*S*,7*S*)-2,7-dibutyroxynonane, of the orange wheat blossom midge, *Sitodiplosis mosellana* (Gries et al. 2000).

The role of (*Z*)-8-heptadecen-2-ol in the pheromone of *R. longicauda* remains to be determined, but traps baited with (2*S*,8*Z*)-2-butyroxy-8-heptadecene or the racemic mixture can be used to monitor this pest.

Acknowledgements This work was funded by the National Science and Technology Support Projects in the 11th Five-year Plan of China (No. 2006BAD15B03, 2006BAD08A03 and 2006BAD02A16-4) and in part by the UK Horticultural Development Council and the Worshipful Company of Fruiterers. We thank Dr. K. M. Harris (former director of International Institute of Entomology, UK) and Prof. Junichi Yukawa (Entomological Laboratory, Faculty of Agriculture, Kyushu University, Japan) for identifying this insect species.

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Identification and Synthesis of the Sex Pheromone of the Madeira Mealybug, *Phenacoccus Madeirensis* Green

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Abstract Two components were identified from aeration extracts of the virgin female Madeira mealybug, *Phenacoccus madeirensis* as *trans*-(1*R*,3*R*)-chrysanthemyl (*R*)-2-methylbutanoate and (*R*)-lavandulyl (*R*)-2-methylbutanoate (with a ratio of 3:1) by a combination of gas chromatography retention time matches, mass spectrometry, and microchemical tests. The structures and chirality of the compounds were confirmed by comparing with synthetic compounds. The synthetic *trans*-(1*R*,3*R*)-chrysanthemyl (*R*)-2-methylbutanoate was highly attractive to males in laboratory bioassays; the synthetic (*R*)-lavandulyl (*R*)-2-methylbutanoate was weakly attractive. No synergistic effect was observed when the mixture of the two compounds was tested.

Keywords Mealybug · *Trans*-(1*R*,3*R*)-chrysanthemyl (*R*)-2-methylbutanoate and (*R*)-lavandulyl (*R*)-2-methylbutanoate · Hemiptera · Pseudococcidae · *Phenacoccus madeirensis*

Introduction

The Madeira mealybug, *Phenacoccus madeirensis* Green, is one of the most polyphagous of all mealybug species, having been reported from more than 42 host families (USDA 2006 and references therein). Its range includes nearly all temperate areas of the world, but it has limited distribution in Australasian and Oriental regions (CAB International 2000). However, it has recently invaded Taiwan (Yeh et al. 2006).

Although parasitoids of the Madeira mealybug have been studied (Chong and Oetting 2008), no work has been done to identify the sex pheromone of this species. Sex pheromones of the related species *Planococcus citri* (Risso) and *Planococcus ficus* Signoret have been identified, and now are widely used in pest management (Moreno et al. 1984; Millar et al. 2002; Walton et al. 2006). Sex pheromones of several other mealybug species have been identified recently, including *Pseudococcus maritimus* (Ehrhorn) (Figadere et al. 2007), *Planococcus minor* (Maskell) (Ho et al. 2007), and *Planococcus kraunhiae* (Kuwana) (Sugie et al. 2008). For a pest as important as *P. madeirensis*, identification of the sex pheromone for use in pheromone-baited monitoring traps would be helpful for detection and management. We report here the identification of two female-specific components of *P. madeirensis* in Taiwan as *trans*-(1*R*,3*R*)-chrysanthemyl (*R*)-2-methylbutanoate [IUPAC name: 2R-Methyl-butanoic acid (1'R, 3'R)-2',2'-dimethyl-3'(2-methyl-propenyl)-cyclopropylmethyl ester] and (*R*)-lavandulyl (*R*)-2-methylbutanoate. The biological activity of the synthetic compounds was verified in laboratory bioassays.

Methods and Materials

Insects A colony of *P. madeirensis* was started from bugs collected from painted copperleaf, *Acalypha wilkesiana* Muell.-Arg, on the campus of National Taiwan University, Taipei, Taiwan in 2006. The mealybugs were reared on sprouted potatoes (Solanaceae: *Solanum tuberosum* L.) in an incubator maintained at 28°C, relative humidity 40–

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80%, and under a photoperiod of 12:12 h (L:D). The identity of the species was confirmed by Dr. S. P. Chen of Department of Applied Zoology and Entomology, Taiwan Agricultural Research Institute, Taichung, Taiwan.

Sprouted potatoes were infested with ovisacs to start new cohorts (10–15 ovisacs for each potato). After 10–14 d, female nymphs were separated from male nymphs by transferring female nymphs with a fine brush to a new sprouted potato to ensure virginity after emergence. The sex of the mealybug could be distinguished by appearance and behaviors of the second-instar nymphs (Chong et al. 2003; Yeh et al. 2006). At this stage, male nymphs become pink and secrete waxy filamentous cocoons, whereas female nymphs remain pale yellow and lack cocoons.

Males of *P. madeirensis* nymphs were separated by the method of Negishi et al. (1980) and Arai (2000) as follows. Male nymphs that crawled off the potato sprouts to pupate on the paper towels that covered the potatoes were collected. The pupae-containing paper towels were put into a container (21.5 cm ID×6.5 cm in depth) and kept in the incubator. Two to 3 days after emergence, males were used in bioassays.

Collection of Pheromone Extracts Sprouted potato tubers infested with virgin females (about 200–350 females per potato) were aerated in a 1,000 ml glass aeration chamber by passing purified air through the chamber. The headspace volatiles were trapped on collectors that consisted of glass tubes packed with activated charcoal (2.5 cm×8 mm ID), and collections continued for 5–7 d. A total of 1,000–2,000 female-day-equivalents (hereafter FDE) of the female-specific compounds were collected per collector. Extracts from aerations of uninfested sprouted potato tubers served as controls.

Analysis of Pheromone Extracts The collectors were eluted with pentane, and the resulting extracts were analyzed by splitless coupled gas chromatography-mass spectrometry (GC-MS) with a ThermoQuest Trace GC interfaced to a Finnigan Trace mass spectrometer (electron impact ionization, 70 eV). The GC oven was held at 40°C for 1 min, programmed at 10°C/min to 250°C, with injector and transfer line temperatures of 200°C and 250°C, respectively. DB-5MS, DB-1, and DB-23 columns (all 30 m×0.25 mm ID, J & W Scientific, Folsom, CA, USA) were used, with helium as carrier gas. The Kovats indices of the unknowns were calculated in relation to straight-chain alkanes. For determination of the absolute configurations of the compounds, a chiral Rt-bDEXsm capillary column (30 m×0.25 mm ID, Restek, Bellefonte, PA, USA) was used. NMR spectra were recorded on Bruker Avance 400 spectrometer.

Isolation of the Pheromone High performance liquid chromatography (HPLC) was employed to isolate the sex pheromone, using an ECOM® Beta 10 Gradient Pump (Prague, Czech Republic), a silica gel column (250× 4.6 mm, 5 μ) (LDC/Milton Roy, Riviera Beach, FL, USA), a Rheodyne Model 7125 sample injector, and a DAD230 photo diode array detector (set at 200–650 nm) (ChromTech, Singapore). Aeration extracts of infested and uninfested potatoes were eluted with a solvent gradient of pentane and diethyl ether (1.0 ml/min), starting with 100% pentane for 2 min, and then decreasing to 0% pentane in 55 min with the addition of diethyl ether. Fractions were collected every 1 min with a fraction collector. The collected fractions were concentrated under a stream of nitrogen and then analyzed by GC/MS.

Hydrolysis of the Unknown The concentrated fraction that contained the unknown (1 μg) was dissolved in 300 μl of ethanol, and two drops of 1 N NaOH were added. The reaction mixture was kept in a water bath at 35°C for about 30 min before 1 N HCl was added to neutralize the solution. The product was extracted with hexane. The hexane solution was dried by passing through a short plug of anhydrous $MgSO_4$. After concentration under a stream of nitrogen, the product was analyzed by GC/MS.

Chemicals Racemic 2-methylbutanoic acid, (*S*)-2-methylbutanoic acid, chrysanthemol (mixture of *trans* and *cis*), lithium aluminum hydride solution in THF, succinic anhydride, (±)-lavandulol, 4-dimethylaminopyridine (DMAP), 1,3-diisopropylcarbodiimide (DIC), and dicyclohexylcarbodiimide (DCC) were obtained from Sigma-Aldrich Co. (Milwaukee, WI, USA). *Trans*-(+)-chrysanthemic acid (CAS # 4638-92-0) was purchased from Acros Organics (Belgium). Pig pancreatic lipase (30.1 U/mg) was purchased from Fluka. Diethyl ether was dried by distillation from sodium-benzophenone ketyl.

Separation of trans- and cis-Chrysanthemol The *trans*- and *cis*- isomers were separated by using semipreparative HPLC on a silica gel column (5 μ, 250×10 mm Luna, Phenomenex) with a solvent gradient of pentane and diethyl ether (2.0 ml/min), starting with 100% pentane for 3 min, and then decreasing to 65% ether in 5 min, to 62% in 80 min, decreasing to 0% pentane in 82 min with the addition of diethyl ether. The *trans*- and *cis*- isomers eluted at around 29 min and 26 min. ^{1}H NMR ($CDCl_3$, 400 MHz) of *trans*-chrysanthemol: δ 0.82 (m, 1H), 1.04 (s, 3H), 1.09 (m, 1H), 1.14 (s, 3H), 1.66 (s, 3H), 1.69 (s, 3H), 3.55 (m, 1H), 3.76 (m, 1H) and 4.85 (d, 1H). ^{1}H NMR ($CDCl_3$, 400 MHz) of *cis*-chrysantheol: δ 1.03 (s, 3H), 1.06 (m, 1H), 1.07 (s, 3H), 1.37 (t, 1H), 1.68 (s, 3H), 1.71 (s, 3H), 3.62 (m, 2H), and 4.95 (d, 1H). The stereo-chemistry was assigned according to the NMR data of the two compounds (Bramwell et al. 1969).

Resolution of (R)-2-Methylbutanoic Acid from Racemic 2-Methylbutanoic Acid Racemic 2-methylbutanoic acid

(58 mg, 0.57 mmole) in 0.5 ml $CHCl_3$ was added to a solution of dicyclohexylcarbodiimide (DCC) (119 mg, 0.57 mmole, in 1 ml $CHCl_3$) cooled in an ice-bath (Kaneda 1986). Then, 70 mg (0.57 mmole) (*S*)-α-methylbenzylamine were added. The solution was heated in a 50°C bath for 1 h. The reaction mixture was cooled and concentrated. Ethyl acetate was added to the residue, and the mixture was filtered. The diastereomeric amide products were in the ethyl acetate filtrate. The diastereomers were separated by using semipreparative HPLC on a silica gel column (5 μ, 250×10 mm Luna, Phenomenex) with a solvent gradient of pentane and diethyl ether (1.0 ml/min), starting with 100% pentane for 2 min, and then decreasing to 0% pentane in 30 min with the addition of diethyl ether. The diastereomers eluted at around 7 min and 8.5 min. Each pure compound was hydrolyzed by refluxing the amide (45 mg) in 1 ml 70% H_2SO_4 for 3 h. After cooling to room temperature, 5 ml water were added, and the mixture was extracted with diethyl ether (10 ml×2). The ether layer was extracted with 2 M NaOH, and the basic aqueous layer was acidified with 1 N HCl, then extracted with diethyl ether. The resolved acid was in the ether layer. After comparison of the resolved acids with authentic (*S*)-2-methylbutanoic acid (from Sigma-Aldrich Co.) on a chiral GC column (r.t. for (*S*)-2-methylbutanoic acid 18.5 min, (*R*)-2-methylbutanoic acid 18.69 min), the compound that eluted first on the HPLC was identified as the amide of (*R*)-2-methylbutanoic acid.

Resolution of (R)-Lavandulol from Racemic Lavandulol The procedure of Zada and Dunkelblum (2006) was followed. Briefly, racemic lavandulol (231 mg, 1.5 mmole), succinic anhydride (300 mg, 3 mmole), and pig pancreatic lipase (340 mg, 30.1 U/mg) were added to 10 ml dry ether, and the mixture was stirred in the room temperature for 24 h. After filtration, the filtrate was diluted with 10 ml of ether and then stirred with 1 M Na_2CO_3 (10 ml) for 30 min. After separation from the aqueous solution, the ether layer was concentrated, yielding 57 mg of (*R*)-lavandulol of 97% optical purity. Absolute configuration of (*R*)-lavandulol was determined according to Zada and Dunkelblum (2006). Because (*S*)-lavandulol will react with the succinic anhydride and become half acid, which was separated from the unreacted (*R*)-lavandulol, (*R*)-lavandulol was in the ether layer. On a chiral GC column, the (*R*)-lavandulol elutes later than the (*S*)-lavandulol (Zhang et al. 2004). The r. t. of the compound in the ether layer was 30.78 min and r.t. of (±)-lavandulol were 29.92 min and 30.78 min, so the compound in the ether layer was identified as (*R*)-lavandulol.

Reduction of Trans-(+)-Chrysanthemic Acid *Trans*-(+)-Chrysanthemic acid (48 mg) was dissolved in 2 ml THF and 0.5 ml of $LiAlH_4$ in THF (2.4 M) was added. After heating at 40°C for 2 h, the mixture was cooled in an ice bath, and quenched by slow, dropwise addition of saturated $NaHCO_3$ (foams!). After gas evolution ceased, 10 ml of diethyl ether were added, and the reaction mixture was stirred at room temperature for 1 h. The ether layer was separated, filtered, dried over $MgSO_4$, and concentrated to yield 37 mg of *trans-(1R,3R)*-chrysanthemyl alcohol.

Preparation of Trans-(1R,3R)-Chrysanthemyl 2-Methylbutanoate and (R)-Lavandulyl (R)-2-Methylbutanoate The *trans-(1R,3R)*-chrysanthemyl alcohol (10 mg, 0.065 mmole), 2-methylbutanoic acid (6.6 mg, 0.065 mmole), 4-dimethylaminopyridine (DMAP) (1.6 mg, 0.013 mmole) and 1,3-diisopropylcarbodiimide (DIC) (9 mg, 0.07 mmole) were combined in dichloromethane (400 μl) and stirred for 1 h at room temperature. After evaporation of the dichloro-

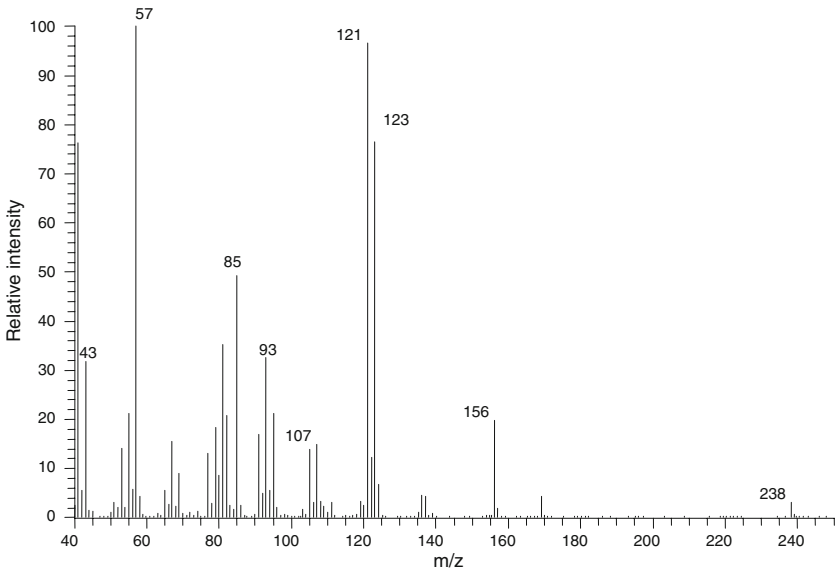


Fig. 1 EI mass spectrum of the female-specific compound A

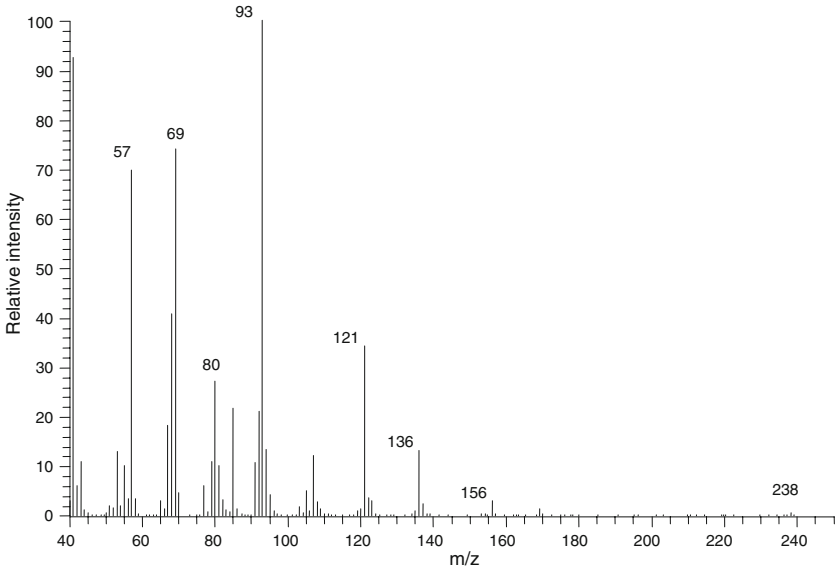


Fig. 2 EI mass spectrum of the female-specific compound B

methane, water was added, and the product was extracted with ether. The compound was purified by semi preparative HPLC on a silica gel column (5 μ, 250×10 mm Luna, Phenomenex) with a solvent gradient of pentane and diethyl ether (1.0 ml/min), starting with 100% pentane for 2 min, and then decreasing to 0% pentane in 30 min with the addition of diethyl ether. ^{1}H NMR ($CDCl_3$, 400 MHz) of *trans-(1R,3R)*-chrysanthemyl 2-methylbutanoate: δ 0.82 (2H, m), 0.89 (3H, t, *J*=7.6 Hz), 1.02 (3H, s), 1.13 (3H, s), 1.15 (3H, d, *J*=6 Hz), 1.45 (2H, quintet, *J*=6.8 Hz), 1.65 (3H, br. s), 1.68 (3H, br. s), 2.34 (1H, sextet, *J*=7.2 Hz), 3.93 (1H,dd, *J*=8.8, 11.6 Hz), 4.29 (1H, dd, *J*=6.8, 11.6 Hz), 4.85 (1H, d, *J*=7.6 Hz). ^{13}C NMR ($CDCl_3$, 75 MHZ): δ 11.6, 16.7, 18.2, 21.5, 22.3, 22.4, 25.6, 26.8, 29.0, 31.0, 41.1, 65.3, 122.9, 133.5, and 176.9. The same procedure was used for the preparation of (*R*)-lavandulyl (*R*)-2-methylbutanoate. ^{1}H NMR ($CDCl_3$, 400 MHz) of (*R*)-lavandulyl (*R*)-2-methylbutanoate: δ 0.87 (3H, t, *J*=7.6 Hz), 1.10 (3H, d, *J*=7.2 Hz), 1.44 (2H, m), 1.58 (3H,s), 1.66 (3H, s), 1.68 (3H, s), 2.06 (2H, m), 2.33 (1H, m), 2.38 (1H, m), 4.03 (2H, d, *J*=6.8 Hz), 4.71 (1H,s), 4.8 (1H, s), 5.05 (1H, t, *J*=6 Hz). ^{13}C NMR ($CDCl_3$, 75 MHZ): δ 11.6, 16.6, 17.8, 19.9, 25.7, 26.7, 28.6, 41.2, 46.2, 65.5, 112.4, 121.7, 132.8, 144.9, and 176.7.

Dynamics of Pheromone Release To determine the diurnal pattern of pheromone release, a cohort of 450 virgin female mealybugs of known age were aerated, and the collectors were changed every 5 h between 19:00 and 15:00, with another collector being used to collect the volatiles between 15:00 and 19:00. Aerations were continued for 3 d. Collectors were eluted as described above, and internal

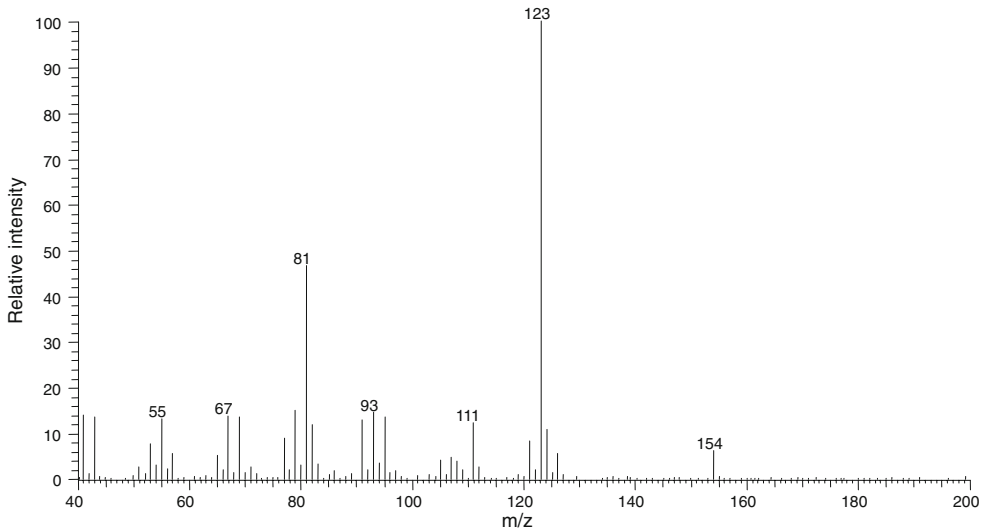


Fig. 3 EI mass spectrum of the hydrolyzed product of compound A

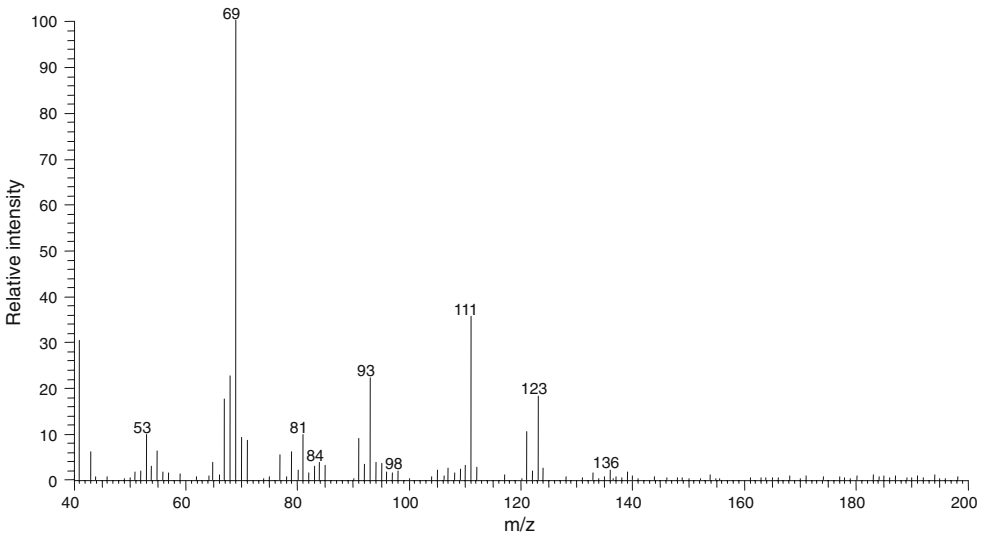


Fig. 4 EI mass spectrum of the hydrolyzed product of compound B

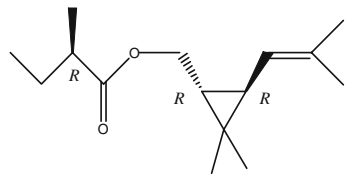
standard (benzophenone, 600 ng) was added to the pentane extract. The amount of the major compound was estimated by comparison of GC peak areas. The amounts of the major compound were recorded in the aeration chamber for a consecutive 24 h period. The data for each time period were combined, and means and standard deviations were calculated. The data are reported as nanograms per hour produced by the 450 female bugs during each time interval. Data were analyzed by ANOVA.

Laboratory Bioassay Bioassays were performed in plastic Petri dishes (14 cm ID; 2 cm in depth) during 19:00–21:00 in a room with fluorescent lights (Tashiro et al. 1969, Lanier et al. 1989, Arai 2000, Zada et al. 2003). Adult males (24–37) that had emerged 1–2 d earlier, together with two pieces of filter paper (2×1 cm) moistened with water, were introduced into the Petri dish about 1–2 h before the bioassay began. Two pieces of filter paper (1×1 cm), one treated with pheromone compound(s) in pentane, and the other with an equivalent amount of pentane as control, were placed in the Petri dish. The numbers of males on each 1 cm^2 paper were recorded 11 times at intervals of 10 min. Treatments included natural extract, synthesized *trans*-(1*R*,3*R*)-chrysanthemyl (*R*)-2-methylbutanoate (compound A), synthesized (*R*)-lavandulyl (*R*)-2-methylbutanoate (compound B) (about 6.7 ng/paper), and a mixture of synthesized compounds A and B in a ratio of 3:1 (6.9 ng: 2.3 ng per paper). The percentage of the males on the filter papers (both the

Table 1 Determination of absolute configuration of the sex pheromone components from the alkaline hydrolyzed acid and alcohols using a chiral GC column

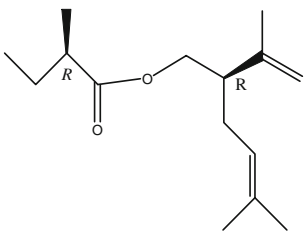
Compounds	Retention times, min[a]
Hydrolyzed product of the aeration extract from females	
2-Methylbutanoic acid	18.69
Lavandulol	30.84
Chrysanthemol	29.1
Synthetic	
(*S*)-2-Methylbutanoic acid	18.50
(*R*)-2-Methylbutanoic acid	18.69
(*S*)-Lavandulol	29.92
(*R*)-Lavandulol	30.78
trans-(1*S*,3*S*)-Chrysanthemol	28.59
trans-(1*R*,3*R*)-Chrysanthemol	28.98
cis-Chrysanthemol	28.55 and 29.84

[a] A Rt-bDEXsm chiral column was used; oven temperature was started at 60°C and then increased at 2°C/min to 180°C



compound A

trans-(*R*,*R*)-chrysanthemyl (*R*)-2-methylbutanoate



compound B

(*R*)-lavandulyl (*R*)-2-methylbutanoate

Fig. 5 Structures of the compound A and compound B

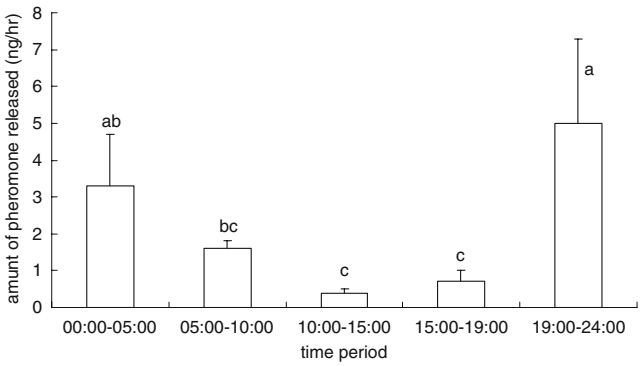


Fig. 6 Diurnal pattern of the amount of chrysanthemyl 2-methylbutanoate (mean ± S. D.) emitted from 450 female *Phenacoccus madeirensi*. Means followed by the same letter were not significantly different at the 5% level (one-way ANOVA). P=0.004

treatment and the control) was calculated for each reading. An average of the 11 readings for each bioassay was recorded as the percentage of male bugs on the treatment or control filter paper. The data were analyzed by t-test.

Results

Analysis Comparison of the extracts of volatiles from mealybug-infested and uninfested potato sprouts revealed two significant insect-produced compounds. The Kovats Index (KI) values of the two compounds were 1727 (compound A) and 1748 (compound B) on the DB-23 column, 1490 (compound A) and 1501 (compound B) on the DB-1 column, and 1492 (compound A) and 1502 (compound B) on the DB-5MS column. The mass spectrum of compound A (Fig. 1) showed significant fragments at *m/z* (%): 57 (100), 81 (35), 85 (48), 93 (32), 107 (14), 121 (98), 123 (76), 136 (4), 156 (19), 169 (4) with a small molecular ion at *m/z* 238 (3). The mass spectrum of compound B (Fig. 2) showed significant fragments at *m/z* (%): 57 (70), 69 (74), 80 (28), 85 (23), 93 (100), 107 (14), 121 (36), 136 (15), 156 (3), also with a small molecular ion at *m/z* 238 (1). The presence of a significant fragmentation ion at *m/z* 85 ($C_4H_9C{\equiv}O^+$) and 136 (M^+-C_4H_9COOH) for both compound A and compound B, suggested that the unknowns could be esters of 5-carbon, saturated carboxylic acids.

The mass spectrum of the hydrolyzed product of compound A (Fig. 3) showed fragment ions with *m/z* (%): 55 (14), 67 (14), 81 (46), 93 (14), 95 (13), 111 (12), 123 (100), and 124 (10), and a molecular ion of *m/z* 154 (6). The mass spectrum of the hydrolyzed product of compound B (Fig. 4) showed fragment ions with *m/z* (%): 53 (10), 55 (8), 69 (100), 81 (10), 93 (24), 111 (36), 121(10), 123 (18), and 136 (4) (M^+-18). The molecular weight of the hydrolyzed product of both compound A and B was 102 mass units less than that of the unknowns, supporting the hypothesis that the unknowns were esters of five-carbon saturated acids. Another compound after hydrolysis showed fragment ions of *m/z* (%): 41 (23), 57 (37), 73 (18), 74 (100), 87(31), and 102 (0.4). Comparison of the mass spectrum of the hydrolyzed products with the NIST mass spectral database, suggested possible structures for the hydrolyzed product of compound A as chrysanthemol, lavandulol for the hydrolysis product of compound B, and the acid part of the ester as 2-methylbutanoic acid.

Both *trans*- and *cis*-chrysanthemol were esterified with 2-methylbutanoic acid, and the esters were compared with compound A from the aeration extract on a DB-23 column. Compound A was confirmed as *trans*-chrysanthemyl 2-methylbutanoate.

The mass spectrum of compound B showed the same fragmentation of the pheromone component as the published spectrum of a component of the pheromone of the pink

Table 2 Percentage of male mealybugs attracted to various treatments in Petri dish bioassays

Treatment	Percentage of males attracted to treatment (mean ± S. D.)	Percentage of males attracted to control (mean ± S. D.)	Difference P value[b]
Compound isolated from mealybug volatiles (6.7 ng[a])	24.5±5.5 (n=5)	3.0±2.2 (n=5)	<0.001
Compound A (*trans*-(1*R*,3*R*)-chrysanthemyl (*R*)-2-methylbutanoate) (6.7 ng)	47.7±9.4 (n=5)	0.7±0.8 (n=5)	<0.001
Compound B ((*R*)-lavandulyl (*R*)-2-methylbutanoate) (6.7 ng)	10.5±6.8 (n=5)	0.9±1.2 (n=5)	0.03
Mixture of compound A and B (3:1, 6.9 ng:2.3 ng)	35.6±9.5 (n=3)	0.2±0.3 (n=3)	0.02
Mixture of compound A and B (3:1, 6.9 ng:2.3 ng)[c]	19.9±6.9 (n=9)	18.8±5.5 (n=9)	0.72

Controls consisted of filter papers treated with solvent

[a] Amount of the major component, chrysanthemyl methylbutanoate (6.7 ng), together with the minor compound lavandulyl methylbutanoate (2.2 ng)

[b] P value after t-test

[c] The control treatment in this experiment is the single compound A (6.9 ng)

hibiscus mealybug, *Maconellicoccus hirsutus* (Zhang, et al. 2004). After comparison of the lavandulyl 2-methylbutanoate with the unknown on DB-23 column, compound B was confirmed as lavandulyl 2-methylbutanoate.

The absolute configuration of the natural pheromone components was unambiguously determined by comparison of retention times of the natural 2-methylbutanoic acid, lavandulol, and chrysanthemol with synthetic isomers on a chiral Rt-bDEXsm capillary column (Table 1). As shown in the table, the pheromones are composed of esters of (*R*)-2-methylbutanoic acid and (*R*)-lavandulol, and *trans*-(1*R*,3*R*)-chrysanthemol, respectively. The structures of the two compounds, A and B are shown in Fig. 5.

Bioassay of Natural Extract and Synthetic Pheromone The diurnal pattern of production of sex pheromone is shown in Fig. 6. Production was the greatest during the scotophase, peaking at 19:00–24:00. Chong et al. (2003) also observed that the males were active during the scotophase, so bioassays were carried out from 19:00 to 21:00. The results for bioassays of the synthesized materials, the compound from volatiles of female mealybugs, and controls are shown in Table 2. Synthetic *trans*-(1*R*,3*R*)-chrysanthemyl (*R*)-2-methylbutanoate (compound A) was highly attractive. (*R*)-Lavandulyl (*R*)-2-methylbutanoate (compound B) and the mixture of the two compounds (in ratio of 3:1) were also attractive to male mealybugs. There was no significant difference between the attractiveness of A and the mixture of compound A and B (last row of Table 2) indicating no additive or synergistic effects between these two compounds.

Concentration of 10 ng to 1×10^{-5}ng of the synthetic compounds and the natural materials were tested in the dose-response experiment and the results are shown in Tables 3 and 4. Within this dosage range, the effectiveness of the compounds increases as the concentration increases. The attractiveness of the synthetic compounds and the natural materials at the dosage of 10^{-3}ng was compared, and the result shown in Table 5 indicates that the synthetic compounds are as effective as the natural material.

Table 3 Percentage of male mealybugs attracted to various dosages of synthetic materials in Petri dish bioassays

Dosage[a]	Percentage of males attracted to treatment (mean ± S. D.) (n=3)	Percentage of males attracted to control (mean ± S. D.) (n=3)	Difference P value[b]
10 ng	31.6±3.8	0.6±0.4	0.004
1 ng	20.0±5.0	0.7±0.6	0.02
1×10^{-1}ng	13.4±0.4	2.1±1.8	<0.001
1×10^{-2}ng	14.4±0.5	1.3±0.6	<0.001
1×10^{-3}ng	7.5±2.5	1.0±0.2	0.04
1×10^{-4}ng	6.3±1.6	3.8±0.9	0.08
1×10^{-5}ng	3.7±2.8	2.3±1.9	0.5

Controls consisted of filter papers treated with solvent only

[a] Amount of the major component, chrysanthemyl methylbutanoate, together with the minor compound lavandulyl methylbutanoate in a ratio of 3:1

[b] P value after t-test

Table 4 Percentage of male mealybugs attracted to various dosages of natural materials in Petri dish bioassays

Dosage[a]	Percentage of males attracted to treatment (mean ± S. D.) (n=3)	ercentage of males attracted to control (mean±S. D.) (n=3)	Difference P value[b]
0.4 ng	28.5±4.6	1.1±1.1	<0.001
1×10^{-2}ng	24.3±1.2	0.9±0.8	<0.001
1×10^{-3}ng	16.4±5.0	3.0±1.4	0.01
1×10^{-4}ng	7.9±0.6	3.8±1.7	0.02
1×10^{-5}ng	3.8±1.3	3.3±2.2	0.7

Controls consisted of filter papers treated with solvent only

[a] Amount of the major component, compound A (together with the minor compound B)

[b] P value after t-test

Discussion

The structures of the major and minor components isolated from female *P. madeirensis* represent further examples of the irregular terpenoid motif of the sex pheromones of pseudococcid species. For example, the sex pheromone of *Pseudococcus viburni* (Millar et al. 2005) is a monoterpene that incorporates a cyclopentane unit. Sex pheromones of *Pseudococcus cryptus* (Arai et al. 2003) and *P. citri* (Bierl-Leonhardt et al. 1981) are monoterpenes with a cyclobutane moiety. The sex pheromone of *P. ficus* (Hinkens et al. 2001), *P. minor* (Ho et al. 2007), and *P. kraunhiae* (Kuwana) (Sugie et al. 2008) are acyclic monoterpenes with the same basic

Table 5 Percentage of male mealybugs attracted to natural and synthetic pheromone components in Petri dish bioassays

Dosage[a]	Percentage of males attracted to natural materials (mean ± S. D.) (n=6)	Percentage of males attracted to synthetic material (mean ± S. D.) (n=6)	Difference P value[b]
1×10^{-3}ng	11.7±3.7	10.6±4.6	0.7

[a] Amount of the major component, compound A (together with the minor compound B)

[b] P value after t-test

carbon skeleton formed from an unusual linkage of two isoprene units. All of the species mentioned above appear to use single components as attractants. It is also interesting to note that the major sex pheromone component we identified here is a monoterpene with a cyclopropane moiety. Chrysanthemol and derivatives have not been found in insects, although they have been isolated from plants (Alexander and Epstein 1975; Gunawardena et al. 2002).

One of the sex pheromone components of pink hibiscus mealybug (PHM), *Maconellicoccus hirsutus*, is (*R*)-lavandulyl (*S*)-2-methylbutanoate (Zhang et al. 2004). Thus, the pheromone components of PHM and Madeira mealybug consist of the same enantiomer of lavandulol, but are esterified with different 2-methylbutanoic acid enantiomers. Zhang et al. (2006) also found that (*R*)-lavandulyl (*R*)-2-methylbutanoate (the compound in the sex pheromone of Madeira mealybug) significantly reduced attraction to (*R*)-lavandulyl (*S*)-2-methylbutanoate (the compound in the sex pheromone of PHM). Because Madeira mealybug produces (*R*)-lavandulyl (*R*)-2-methylbutanoate, it should not cross-attract PHM, thus helping to assure the reproductive isolation of the two species.

The related San Jose scale insect, *Quadraspidiotus perniciosus* (Comstock), produces three components, 7-methyl-3-methylene- 7-octen-1-yl propanoate, (*Z*)-3,7-dimethyl-2,7-octadien-1-yl propanoate, and (*E*)-3,7-dimethyl-2,7- octadien-1-yl propanoate, with each compound being active as a single component, and no apparent synergism in blends of the components (Anderson et al. 1981). However, in the PHM, the two-component blend is needed to attract male PHM.

In summary, we identified two compounds from the aeration extracts of the female Madeira mealybug, *P. madeirensis,* a major pest of economically important crops. Females produce the two components in a 3:1 blend. Compound B is only weakly attractive vs. a blank control, and it does not act additively or synergistically with the major component. Thus, it appears that the active pheromone is a single compound, as in most other mealybugs (except PHM). The importance of the chirality of the components and the role of compound B will be studied further in our laboratory.

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Male-Produced Aggregation Pheromones of the Cerambycid Beetles *Xylotrechus colonus* and *Sarosesthes fulminans*

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Abstract Adults of both sexes of the cerambycid beetles *Xylotrechus colonus* (F.) and *Sarosesthes fulminans* (F.) were attracted to odors produced by male conspecifics in olfactometer bioassays. Analyses of headspace volatiles from adults revealed that male *X. colonus* produced a blend of (*R*)- and (*S*)-3-hydroxyhexan-2-one and (2*S*,3*S*)- and (2*R*,3*R*)-2,3-hexanediol, whereas male *S. fulminans* produced (*R*)-3-hydroxyhexan-2-one and (2*S*,3*R*)-2,3-hexanediol. All of these compounds were absent in the headspace of females. Two field bioassays were conducted to confirm the biological activity of the synthesized pheromones: (1) enantiomerically enriched pheromone components were tested singly and in species-specific blends and (2) four-component mixture of racemic 3-hydroxyhexan-2-one plus racemic 2-hydroxyhexan-3-one and the four-component blend of the stereoisomers of 2,3-hexanediols were tested separately and as a combined eight-component blend. In these experiments, adult male and female *X. colonus* were captured in greatest numbers in traps baited with the reconstructed blend of components produced by males, although significant numbers were also captured in traps baited with (*R*)-3-hydroxyhexan-2-one alone or in blends with other compounds. Too few adult *S. fulminans* were captured for a statistical comparison among treatments, but all were caught in traps baited with lures containing (*R*)-3-hydroxyhexan-2-one. In addition to these two species, adults of two other species of cerambycid beetles, for which pheromones had previously been identified, were caught: *Neoclytus a. acuminatus* (F.) and its congener *Neoclytus m. mucronatus* (F.). Cross-attraction of beetles to pheromone blends of other species, and to individual pheromone components that are shared by two or more sympatric species, may facilitate location of larval hosts by species that compete for the same host species.

Keywords Cerambycidae · Wood-borer · Insect communities · Sex pheromone · Aggregation pheromone · (*R*)-3-hydroxyhexan-2-one

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Introduction

Aggregation or sex pheromones produced by males have been identified for 12 species of longhorned beetles of the subfamily Cerambycinae (Coleoptera: Cerambycidae; Lacey et al. 2004, 2007b; Hanks et al. 2007; Ray et al. 2009a, b, and references therein). Pheromone components of most of these species are typically six to ten carbons long with hydroxyl or carbonyl groups at C_2 and C_3 (Lacey et al. 2004, 2007b; but see Lacey et al. 2008; Hanks et al. 2007; Ray et al. 2009a, b). In addition, minor components that are different from this general structural motif may be important synergists in the pheromone (Fettköther et al. 1995; Reddy et al. 2005; Lacey et al. 2008).

In this article, we describe investigations of the chemical ecology of two sympatric and synchronic cerambycine

species of the tribe Clytini, X*ylotrechus colonus* (F.) and *Sarosesthes fulminans* (F.). Larvae of both species develop in woody tissues of stressed, moribund, and damaged (often wind-thrown) trees of a variety of hardwood species (especially of the genera *Caryae* and *Quercus*; for general biology, see Linsley 1964). Both species are native to eastern North America. Adults are active in spring and summer and are crepuscular, flying from ~16:00 to 22:00 h (ESL, personal observation). Adult male and female *X. colonus* commonly aggregate, in numbers that may exceed 30 individuals per group, on larval hosts. Adult *S. fulminans* may be present on the same larval hosts, but in much smaller numbers (ESL, personal observation). Adult male *X. colonus* and *S. fulminans* have pores on the surface of the prothorax that are characteristic of pheromone production in other species of cerambycines (Ray et al. 2006). Male *X. colonus* sometimes also display a characteristic posture that has been associated with pheromone release in other cerambycine species (the "push-up stance"; Lacey et al. 2007a).

We tested the hypotheses that male *X. colonus* and *S. fulminans* produce aggregation pheromones that conform to the structural motif of other cerambycines and that complete blends of synthetic pheromone components are more attractive to beetles than individual components. We also tested the responses of both species to traps baited with blends of all regio- and stereoisomers of the main pheromone components. These blends are more economical to synthesize than enantiomerically enriched pheromone components and have proven effective as attractants for other cerambycine species (Hanks et al. 2007).

Methods and Materials

Source of Insects Adult *X. colonus* and *S. fulminans* were collected by hand on 6 June 2004 from a wind-thrown white oak, *Quercus alba* L., at Allerton Park (Piatt Co., IL, USA), a 600-ha mixed hardwood forest owned by the University of Illinois. Adults were housed individually in the laboratory (12:12 h L:D, ~20°C, ~50% RH) in cylindrical cages, constructed of aluminum window screen, with plastic Petri dishes at top and bottom, and provided with 10% sucrose in water. Beetles used in bioassays were active and apparently healthy.

Olfactometer Bioassays We tested for volatile attractants in both species by using a horizontal glass Y-tube olfactometer (6 cm diameter, main tube 26 cm long, arm length 22 cm, and 70° angle between arms). We conducted olfactometer studies outdoors in partial shade because all cerambycine species that we have bioassayed to date did not respond in Y-tube olfactometer bioassays conducted under laboratory conditions (see Lacey et al. 2004, 2007b, 2008). A 2-L plastic chamber, containing a cylinder of aluminum screen for a perch, was attached to each arm of the Y-tube. When bioassaying *X. colonus*, one chamber held six males and the other held six females. Only three *S. fulminans* of each sex were used as odor sources in their respective bioassay, because this species was less abundant. Ambient air was pulled through the olfactometer (air speed 2.5 m s^{-1}) with a 0.75 kW vacuum cleaner connected to a variable voltage power supply. For each trial, a beetle was released at the base of the Y-tube and allowed 10 min to respond (crossing a line 18 cm down one arm) to an odor source. Chambers were alternated between Y-tube arms every three trials to control for positional bias. The chambers and olfactometer were washed with unscented laboratory detergent (Alconox® powder, Alconox, Inc., NY, USA) and rinsed with acetone each day. We bioassayed 20 *X. colonus* of each sex between 17:00–20:00 h on 9, 10, and 13 June 2004 and 13 female and seven male *S. fulminans* during the same hours on 11 and 12 June 2004 (skies clear, air temperatures ~24–30°C). For each species, numbers of each sex responding to treatments were compared with the χ^2 goodness-of-fit test corrected for continuity (Sokal and Rohlf 1995).

Identification of Pheromone Components Volatile compounds produced by adult *X. colonus* and *S. fulminans* were collected by placing five adult females and males in separate glass vacuum traps (0.3 L, manufactured by the glass shop, School of Chemical Sciences, UIUC) that were lined with aluminum screen to provide perches. A glass tube (6 cm long×9.5 o.d.×4 mm i.d.) containing 100 mg of 80/100 mesh SuperQ® (Alltech Associates, Deerfield, IL, USA) was attached to one nipple of each chamber with an 8-cm long section of Teflon® tubing. Charcoal-purified air was pulled through the apparatus with a water aspirator (1 L min^{-1}). Males and females of each species were aerated simultaneously on a laboratory windowsill from 16:00 to 22:00 h: *X. colonus* on 11, 12, and 13 June and *S. fulminans* on 14, 15, and 16 June 2004. We selected these time periods for collecting volatiles from beetles because they corresponded to activity periods in the field (ESL, personal observation). Collectors were eluted into silanized glass vials (Cat. #27114, Supelco®, Bellefonte, PA, USA) with three 0.5-ml aliquots of methylene chloride. The resulting extracts were analyzed with a Hewlett-Packard® (Sunnyvale, CA, USA) 6890 gas chromatograph (GC) coupled to a 5973 mass selective detector (MS). The GC was fitted with a DB5-MS column (30 m×0.25 mm, 25 µm film thickness; Agilent Technologies, Santa Clara, CA, USA) and programmed from 40°C (held for 1 min) to 250°C at 10°C min^{-1} (held for 15 min), with an injector temperature of 250°C. Injections were made in the splitless mode. When it became clear that one or more of the

compounds in the extract might be thermally unstable (see Leal et al. 1995; Lacey et al. 2007b), the injector temperature was lowered to 100°C, and the temperature program was changed to 20°C (held for 2 min) to 250°C at 10°C min^{-1} (held for 15 min). Absolute configurations of the insect-produced compounds were determined by analysis of aliquots on a Cyclodex-B GC column (30 m× 0.25 mm, 0.25 micron film thickness, J&W Scientific, Folsom CA, USA) with the GC programmed from 50°C (held for 1 min) to 200°C at 5°C min^{-1}, injector 100°C, detector 200°C. Identifications of peaks were confirmed by coinjections of extracts with authentic standards.

Field Bioassays of Synthetic Pheromone In Experiment 1, bioassays of reconstructed blends of male-specific volatiles for each of the two species, as well as of the individual components, were conducted on 9 days between 11 June and 27 July 2006 and 5 days between 29 May and 14 June 2007. Bioassays were carried out at Allerton Park and Brownfield Woods (Champaign Co., IL; skies clear to partly cloudy, maximum air temperatures ~22–30°C, wind speed ~8–20 kph), the latter a 26-ha mixed deciduous forest owned by the University of Illinois. We used two different sites because we observed that trap catches of cerambycine species at individual sites decline over time, possibly because trapping depletes local populations (ESL, unpublished data). Both of the sites had populations of *X. colonus* and *S. fulminans* (ESL, personal observation) and replications of the experiment were assigned randomly to sites.

Traps were black cross-vane flight intercept panel traps (1.2 m tall×0.30 m wide, Intercept™, model PT, APTIV, Inc., Portland, OR, USA). Traps were suspended from hangers constructed of 1.27 cm i.d. PVC pipe (D1785, Schedule 40, Charlotte Pipe and Foundry Co., Charlotte, NC, USA), with a 1.5 m long upright connected with a T fitting to a 20-cm long arm with a loop of wire at the end to which the trap was attached. The upright was mounted on a 1.5 m section of 1.27 cm diameter steel reinforcing bar driven into the ground. Traps were positioned 10 m apart in a straight line approximately perpendicular to the prevailing wind direction. Traps were set up at ~14:00 h and beetles were removed from traps at ~11:00 h the following day.

Enantiomerically enriched hydroxyhexanones (94% ee; Lacey et al. 2007a, b) and hexanediols (>98% stereoisomerically pure, Lacey et al. 2008) were synthesized as previously described. Each treatment was dissolved in 1 ml of absolute ethanol. We randomly assigned each of the following nine treatments to traps: (1) *X. colonus* blend (see "Results"): (*R*)-3-hydroxyhexan-2-one (8 mg), (*S*)-3-hydroxyhexan-2-one (1.25 mg), (2*R*,3*R*)-2,3-hexanediol (2.5 mg), and (2*S*,3*S*)-2,3-hexanediol (0.5 mg); (2) *S. fulminans* blend (see "Results"): (*R*)-3-hydroxyhexan-2-one (8 mg) and (2*S*,3*R*)-2,3-hexanediol (1.25 mg); (3) (*R*)-3-hydroxyhexan-2-one (8 mg); (4) (*S*)-3-hydroxyhexan-2-one (8 mg); (5) (2*S*,3*S*)-2,3-hexanediol (8 mg); (6) (2*R*,3*R*)-2,3-hexanediol (8 mg); (7) (2*S*,3*R*)-2,3-hexanediol (8 mg); (8) ethanol control (1 ml); and (9) blank control (empty emitter).

Pheromones were loaded into release devices that consisted of modified 1.5-ml microcentrifuge tubes (#05-406-16; Fisher Scientific, Pittsburg, PA, USA). A section of polyethylene tubing (25 mm long, 4 mm o.d., 2 mm i.d.) was inserted through a 4 mm hole drilled through the tube cap and cemented in place with quick setting epoxy glue such that 8 mm of tubing extended beyond the cap. A 5-cm long section of pipe cleaner (cotton fiber and steel wire, 2.5 mm diameter) was inserted through the tubing such that one end reached the bottom of the microcentrifuge tube and the other end extended 5 mm beyond the end of the polyethylene tubing (modified from Hanks et al. 2007). Ethanol is an efficient carrier of hydroxyhexanones and hexanediols (Hanks et al. 2007) and the lures released ~1 ml of ethanol solution over the course of 1 day under field conditions (unpublished data). Lures were hung with wire in the open central slot of panel traps.

Differences among treatments in numbers of beetles captured (sexes combined) were tested with the nonparametric Friedman's test (blocked by site and day; PROC FREQ with CMH option, SAS Institute, 2001) because assumptions of analysis of variance were violated by heteroscedasticity (Sokal and Rohlf 1995). Differences between pairs of means were tested with the REGWQ means-separation test to control maximum experiment-wise error rates (SAS Institute, 2001). Replications that captured fewer than two beetles overall (N=9) were excluded from the statistical analyses for a given species. Differences between numbers of females and males captured within treatments were tested with the χ^2 goodness-of-fit test (Sokal and Rohlf 1995).

We conducted a further trial ('Experiment 2') to test the responses of *X. colonus* and *S. fulminans* to blends of stereoisomers of the chemicals to determine whether unnatural regio- and/or stereoisomers had an inhibitory effect. This bioassay was conducted on 25 days between 13 June–31 August 2006 and 18 days between 5 May–15 August 2007 at Allerton Park and Brownfield Woods using the methods described above. Treatments were as follows (compounds synthesized as described in Hanks et al. (2007) and diluted to 1 ml with absolute ethanol): (1) ~1:1:1:1 blend of enantiomers of 3-hydroxyhexan-2-one and 2-hydroxyhexan-3-one (100 mg total load, 25 mg of each isomer; henceforward referred to as "generic hydroxyhexanones"); (2) ~1:1:1:1 blend of all four stereoisomers of 2,3-hexanediol (100 mg total load, 25 mg of each isomer; henceforward "generic hexanediols"); (3) 1:1 combination

of generic hydroxyhexanones and generic hexanediols (200 mg total load); (4) ethanol control (1 ml); and (5) blank control (empty emitter).

The mixtures were loaded into lures and differences among treatments in numbers of beetles captured per species were tested as in the previous bioassay. Replications that captured fewer than two beetles of a given species were excluded from statistical analysis (N=28). On days when enantiomerically enriched pheromones and generic pheromones were bioassayed simultaneously, trap lines were separated by >1 km.

Results

Olfactometer Bioassays In olfactometer bioassays, 17 female *X. colonus* were attracted to odors emitted by live males, compared to only two that responded to odors of live females (χ^2=11.8, P< 0.001). Male *X. colonus* showed a similar response, with 16 responding to odors of live males compared to only one responding to odors from live females (χ^2=13.2; P< 0.001). Both sexes of *S. fulminans* were attracted to odors produced by male conspecifics, with 12 females responding to males and only one responding to females (χ^2=9.31; P< 0.01) and all seven males responding to males (χ^2=7.00; P< 0.05). These findings confirmed that males of both species produce volatile pheromones that attract both sexes.

Identification of Pheromone Components GC-MS analyses of volatiles collected from male *X. colonus* revealed two major peaks in the total ion chromatogram that were absent in the analogous headspace of females. The retention time and mass spectrum of the larger peak (base peak at *m/z* 55, other significant fragments at *m/z* 43 [78%], 73 [53%], and 45 [26%]) matched those of a synthetic standard of 3-hydroxyhexan-2-one. The retention time and mass spectrum of the smaller peak (base peak at *m/z* 55, other significant fragments at *m/z* 75 [10%], 73 [61%], 72 [34%], 45 [26%], and 43 [35%]) matched those of a synthetic standard of 2,3-hexanediol. The stereoisomeric compositions of the insect-produced compounds were determined to be ~70% (*R*)- and 10% (*S*)-3-hydroxyhexan-2-one and 17% (2*S*,3*S*)- and 3% (2*R*,3*R*)-2,3-hexanediol by analysis on the Cyclodex-B column, with the enantiomers being resolved to baseline (for elution order of all hydroxyketones and 2,3-hexanediols, see Ray et al. 2009b). Analogous collection and analysis of headspace volatiles from both sexes of *S. fulminans* revealed that males produced ~83% (*R*)-3-hydroxyhexan-2-one and 17% (2*S*,3*R*)-2,3-hexanediol as the only detectable male-specific components (identified as described above).

Field Bioassays of Synthetic Pheromone In Experiment 1, testing chiral synthetic pheromone components, traps captured 79 adult *X. colonus* of both sexes, but only ten adult *S. fulminans* (Table 1). Treatments differed significantly in numbers of *X. colonus* captured (Fig. 1; Friedman's $Q_{8,79}$=52.3, P<0.001). Only traps baited with (*R*)-3-hydroxyhexan-2-one alone or in a blend (i.e., the *X. colonus* and *S. fulminans* blends) were significantly attractive compared to ethanol and blank controls (Fig. 1). The blend

Table 1 Results of experiment 1

	Xylotrechus colonus		*Sarosesthes fulminans*		*Neoclytus a. acuminatus*	
Treatment	♀	♂	♀	♂	♀	♂
(1) *X. colonus* blend	17	13	2	1	4	3
(2) *S. fulminans* blend	9	9	3	2		
(3) (*R*)-3-hydroxyhexan-2-one	10	7	2			
(4) (*S*)-3-hydroxyhexan-2-one	5	3				
(5) (*R*,*R*)-2,3-hexanediol	1					
(6) (*S*,*R*)-2,3-hexanediol					1	1
(7) (*S*,*S*)-2,3,-hexanediol	3				22	14
(8) Ethanol control	1	1				
(9) Blank control						
Total	46	33	7	3	27	18

Numbers of cerambycid beetles of three species caught in traps baited with enantiomerically enriched candidate pheromone components (N=9 replicates for *Xylotrechus colonus* and *Sarosesthes fulminans*; N=10 replicates for *N. a. acuminatus*). The blend of components specific to *X. colonus* contained (*R*)- and (*S*)-3-hydroxyhexan-2-one and (2*R*,3*R*)- and (2*S*,3*S*)-2,3-hexanediol. The *S. fulminans* blend contained (*R*)-3-hydroxyhexan-2-one and (2*S*,3*R*)-2,3-hexanediol. Treatment numbers correspond to treatments in Fig. 1. Sex ratio of total numbers within species were not significantly different from 1:1 (χ^2, P>0.05).

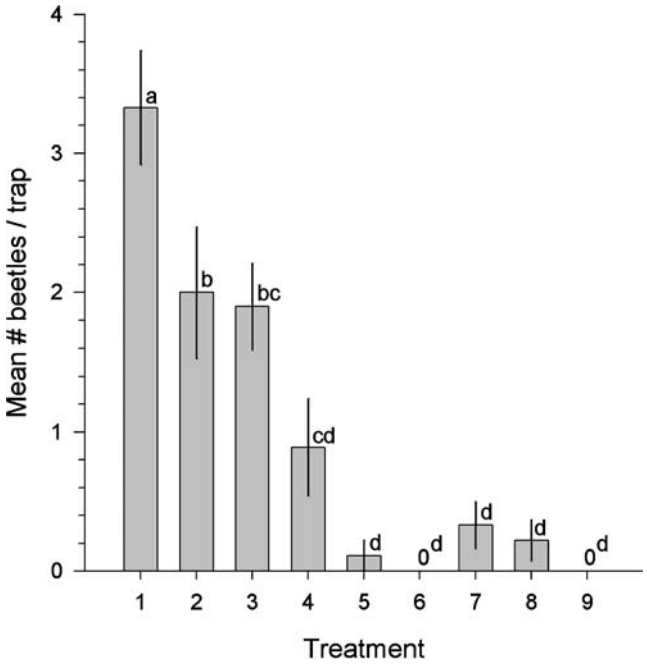

Fig. 1 Mean (±SE) numbers of adult *Xylotrechus colonus* (N=9 trials) captured per trap (sexes combined) with respect to composition of the lure (N=9 trials). The blend of components specific for *X. colonus* contained (*R*)- and (*S*)-3-hydroxyhexan-2-one with (2*R*,3*R*)- and (2*S*,3*S*)-2,3-hexanediol, the *Sarosesthes fulminans* blend contained (*R*)-3-hydroxyhexan-2-one and (2*S*,3*R*)-2,3-hexanediol. Treatment numbers correspond to treatments in Table 1: (*1*) *X. colonus* blend, (*2*) *S. fulminans* blend, (*3*) (*R*)-3-hydroxyhexan-2-one, (*4*) (*S*)-3-hydroxyhexan-2-one, (*5*) (2*R*,3*R*)-2,3-hexanediol, (*6*) (2*S*,3*R*)-2,3-hexanediol, (*7*) (2*S*,3*S*)-2,3-hexanediol, (*8*) ethanol control, and (*9*) blank control. Means with different letters are significantly different (REGWQ test; P<0.05)

of pheromone components specific for *X. colonus* captured the greatest number of beetles (Fig. 1). Too few adult *S. fulminans* were captured for statistical analysis of treatments; however, all ten beetles were captured with lures that contained the most abundant component of its volatile collection, (*R*)-3-hydroxyhexan-2-one (Table 1).

During Experiment 1, we also trapped adults of another cerambycine species, *Neoclytus acuminatus acuminatus* (F.) (Table 1), the male-produced pheromone of which is composed solely of (2*S*,3*S*)-2,3-hexanediol (Lacey et al. 2004). Treatments differed in numbers of *N. a. acuminatus* captured (Friedman's $Q_{8,45}$=64.2, P<0.001, ten replicates), with only traps baited with (2*S*,3*S*)-2,3-hexanediol catching significantly more beetles (3.60±0.54, sexes combined) than ethanol and blank controls (means=0 for both).

In Experiment 2, testing the generic blends of pheromone stereoisomers, we captured 111 adult *X. colonus* of both sexes (Table 2) but only eight *S. fulminans*. Treatments again differed in numbers of adult *X. colonus* captured (Fig. 2, top; Friedman's $Q_{4,111}$=70.98, P<0.001), with significantly greater numbers in traps baited with generic hydroxyketones, alone or in combination with generic hexanediols, than in solvent or blank controls. No adult *X. colonus* were captured in traps baited with only the generic hexanediols. Again, too few adult *S. fulminans* were captured for statistical analysis, although all were captured in traps with lures that contained generic hydroxyketones (Table 2).

In Experiment 2, traps baited with generic pheromone lures also captured 121 adult *N. a. acuminatus* of both sexes, as well as 50 adult males and females of a congener, *Neoclytus m. mucronatus* (F.) (Table 2). The male-produced pheromone of *N. m. mucronatus* is composed of only (*R*)-3-hydroxyhexan-2-one (Lacey et al. 2007b). Treatments differed in numbers of *N. a. acuminatus* and *N. m. mucronatus* captured (Fig. 2; Friedman's $Q_{4,121}$=89.3, P< 0.001; $Q_{4,50}$=36.3 P<0.001, respectively). Significantly more *N. a. acuminatus* were captured in traps baited with generic hexanediols, alone or in combination with hydrox-

Table 2 Results of experiment 2

	Xylotrechus colonus		*Sarosesthes fulminans*		*Neoclytus a. acuminatus*		*Neoclytus m. mucronatus*	
Treatment	♀	♂	♀	♂	♀	♂	♀	♂
(1) Hydroxyhexanones	29	30	3	2			22	13
(2) Hexanediols					27	38		1
(3) Ketones + Diols	23	24	2	1	22	32	9	5
(4) Ethanol control	2	3				2		
(5) Blank control								
Total	54	57	5	3	49	72	31	19*

Numbers of cerambycid beetles of four species caught in traps baited with generic pheromone blends (N=31, 28, 28, and 18 replicates for *Xylotrechus colonus*, *Sarosesthes fulminans*, *N. a. acuminatus*, and *N. m. mucronatus*, respectively). The blend of hydroxyhexanones contained equal amounts of (*R*)- and (*S*)-3-hydroxyhexan-2-one and (*R*)- and (*S*)-2-hydroxyhexan-3-one, and the blend of hexanediols contained approximately equal amounts of (2*R*,3*R*)- (2*S*,3*S*)- (2*R*,3*S*)- and (2*S*,3*R*)-2,3-hexanediol. The treatment designated 'Ketones + Diols' contained equal amounts of the blends of hydroxyhexanones and hexanediols. Treatments are numbered to correspond to treatments in Fig. 2.

*P<0.05 (χ^2) next to total number of males indicates sex ratio of beetles captured within species that is significantly different from 1:1

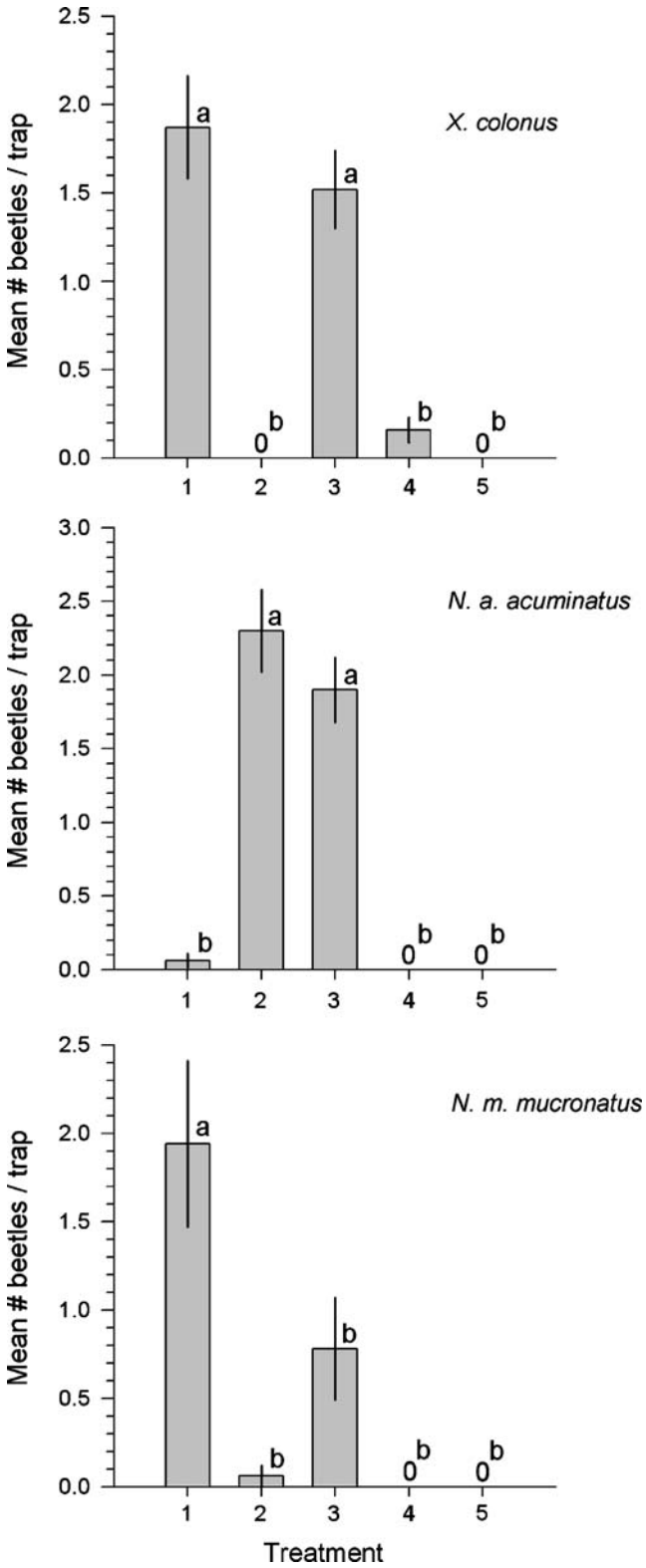

Fig. 2 Mean (±SE) numbers of adults (sexes combined) of three species of cerambycid beetles captured with respect to composition of the lure: *top*, *Xylotrechus colonus* (*N*=31 trials); *middle*, *Neoclytus a. acuminatus* (*N*=28 trials); and *bottom*, *Neoclytus m. mucronatus* (*N*=18 trials). Treatment numbers correspond to treatments in Table 2: (*1*) generic hydroxyhexanones; (*2*) generic hexanediols; (*3*) 1:1 mixture of generic hydroxyhexanones and hexanediols; (*4*) ethanol control; and (*5*) blank control. Means with different letters are significantly different (REGWQ test; $P<0.05$)

yhexanones (Fig. 2, middle), than in control traps or traps baited with generic hydroxyketones. Conversely, adult *N. m. mucronatus* were only significantly caught in traps baited with generic hydroxyhexanones (Fig. 2, bottom).

In both field bioassays, traps baited with the synthetic pheromone components also captured small numbers (total catch <8 individuals) of an additional 11 species of cerambycids, including seven species in the subfamily Cerambycinae (*Anelaphus pumilus* [Newman], *Cyrtophorus verrucosus* [Olivier], *Knulliana cincta* [Drury], *Neoclytus caprea* [Say], *Neoclytus scutellaris* [Olivier], *Phymatodes amoenus* [Say], *Xylotrechus convergens* LeConte), three species of the subfamily Lamiinae (*Liopinus alpha* [Say], *Psenocerus supernotatus* [Say], and *Urographis fasciatus* [Degeer]), and one species of subfamily Parandrinae (*Neandra brunnea* [F.]). Traps captured few insects other than the species reported here.

Discussion

Attraction of both sexes of *X. colonus* and *S. fulminans* to odors from conspecific males in olfactometer bioassays provided the first evidence that males of both species produce aggregation pheromones. Traps baited with synthetic pheromone captured similar numbers of male and female *X. colonus*, confirming that males produce an aggregation pheromone (Tables 1 and 2). The small number of *S. fulminans* captured is consistent with its scarcity in the habitats where we conducted our bioassays (ESL, personal observation). The structure of the pheromone components of *X. colonus* and the putative pheromone compounds of *S. fulminans* are consistent with the ketone/diol structural motif of other cerambycine species (see "Introduction") and provide further support for the hypothesis that this structural motif has been conserved within the Cerambycinae (see Lacey et al. 2004, Hanks et al. 2007). Nevertheless, recent studies have shown that some cerambycine species have pheromone components of entirely different chemical classes (Hanks et al. 2007; Lacey et al. 2008; Ray et al. 2009a). Our study also provides further evidence that the presence of gland pores on the prothorax of males (in *X. colonus*, *S. fulminans*, *N. a. acuminatus*, and *N. m. mucronatus*; Ray et al. 2006, Lacey et al. 2007b) and calling behavior of males (in *X. colonus*, *N. a. acuminatus*, and *N. m. mucronatus*; Lacey et al. 2007a, b) reliably indicate that volatile pheromones play a role in mate location in cerambycine species.

In both field experiments, all treatments that contained (*R*)-3-hydroxyhexan-2-one attracted significant numbers of adult *X. colonus* (Figs. 1 and 2 (top)). However, *X. colonus* were caught in greatest numbers in traps baited with its species-specific blend (Fig. 1), suggesting that the full blend is necessary for optimal attraction. In the absence of (*R*)-3-hydroxyhexan-2-one, adult *X. colonus* responded weakly, or did not respond (Fig. 1) to the minor components of the blend produced by males [including (*S*)-3-hydroxyhexan-2-one and (2*R*,3*R*)- and (2*S*,3*S*)-2,3-hexanediols]. Unnatural isomers apparently did not inhibit attraction of *X. colonus* to (*R*)-3-hydroxyhexan-2-one, as evidenced by the significant number of beetles captured in traps baited with the *S. fulminans* lure or the generic hydroxyketones (Figs. 1 and 2 (top)). Attraction of adult males and females of a congener, *Xylotrechus nauticus* (Mannerheim), to synthetic pheromones also is not inhibited by unnatural isomers (Hanks et al. 2007).

(*R*)-3-Hydroxyhexan-2-one now has been shown to be an important component of male-produced pheromones of 14 species of cerambycine beetles (including *X. colonus* and possibly *S. fulminans*; see Hanks et al. 2007). Overlap in pheromone composition among sympatric cerambycine species can result in cross-attraction (Hanks et al. 2007). Cross-attraction was evident in the present study, with both sexes of *X. colonus* caught in traps baited with treatments representative of reconstructed blends of the pheromone produced by *S. fulminans* (Fig. 1), as well as the other species caught in traps baited with compounds identified in the two focal species of this study. *X. colonus*, *S. fulminans*, *N. a. acuminatus*, and *N. m. mucronatus* are sympatric, overlap in phenology, and share larval hosts (Linsley 1964). In fact, adults of all four species may aggregate on the same downed host tree (ESL, personal observation) and adult *X. colonus* and *N. m. mucronatus*, at least, are attracted to odors emanating from dying hosts (Ginzel and Hanks 2005). Larvae of all four species require hosts that are severely weakened or moribund (Linsley 1964) and competition for these resources (with other insect species and saprophytes), results in rapid degradation of hosts (Hanks 1999). Species that rely on such ephemeral hosts may be under selective pressure to locate hosts quickly and oviposit (Hanks 1999). Thus, these species may exploit the pheromones of other species in their feeding guild as kairomones. Closely related species of bark beetles (Curculionidae: Scolytinae), as well as closely related species of sap beetles (Nitidulidae), share pheromone components, respectively, resulting in cross-attraction among species (e.g., Zilkowski and Bartelt 1999; Haberkern and Raffa 2003). Perhaps not coincidentally, males of these bark and sap beetle species also produce aggregation pheromones (Zilkowski and Bartelt 1999; Haberkern and Raffa 2003) such as those produced by male cerambycine beetles.

The responses of beetles of three species, *X. colonus*, *N. a. acuminatus*, and *N. m. mucronatus*, to generic lures (Table 2, Fig. 2) again demonstrates that these blends could be used to collect live specimens of various cerambycine species, for analysis and identification of the pheromone blends that they actually produce. In this context, even weak attraction to generic blends may be sufficient for collecting adults for pheromone identification or even monitoring the distribution and phenology of economically important species of interest for which a pheromone has yet to be identified.

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Natural Ligands of Porcine Olfactory Binding Proteins

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Abstract Knowledge of endogenous ligands of olfactory binding proteins is a prerequisite for studying their role in odor and pheromone transduction. Here, we report the extraction, derivatization, and characterization by gas chromatography-mass spectrometry of the natural ligands of pig, *Sus scrofa* (L.), Von Ebner's Gland protein (VEG) and odorant binding protein (OBP). We identified two isoforms (VEG1 and VEG2), which differed only by the linkage of an *O*-N-acetylglucosamine (*O*-GlcNac) group on VEG1. The natural ligands of VEG1 were characterized as two isomers of testosterone, whereas ligands of VEG2 and OBP were fatty acids or their derivatives. Our findings suggest that the binding specificity of VEG1 for steroids is governed by the presence of an *O*-GlcNac moiety on the protein. This specificity was confirmed by the binding of radiolabeled testosterone only by VEG1 in an in-gel binding assay. This is the first evidence for a post-translational modification in the process of odorant discrimination by olfactory binding proteins.

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Keywords Endogenous ligand · Fatty acid · MALDI-TOF mass spectrometry · Odorant binding protein · Olfaction · *O*-N-acetylglucosamine · *Sus scrofa* · Testosterone · Tryptic digestion · Von Ebner's Gland protein

Introduction

Olfactory binding proteins are small soluble proteins secreted in a variety of tissues and organs related to chemical communication (Tegoni et al. 2000). They are probably an adaptation to terrestrial life, as members of this protein group seem to be absent in fish, but widespread in insects, mammals, and reptiles. Their physiological role depends on their localization: 1) binding and transport of odors and pheromones in most tissues and fluids, as odorant binding proteins (OBP) of saliva (Marchese et al. 1998) and amniotic fluid (Guiraudie-Capraz et al. 2005), hamster aphrodisin (Singer et al. 1986), or tear lipocalin in mammals (Garibotti et al. 1995; Redl 2000), and 2) odor and pheromone transduction in olfactory organs, as pheromone-binding proteins (PBP) in insects (Xu et al. 2005). This latter physiological role is better understood in insects than in mammals, where the involvement of olfactory binding proteins in odor discrimination remains to be elucidated. Binding to specific ligands has only been demonstrated for the sub-class of pheromone-binding proteins such as Major Urinary Proteins (MUP) of rodents (Robertson et al. 1993), salivary lipocalin (SAL) in the pig, *Sus scrofa* (L.) (Marchese et al. 1998; Scaloni et al. 2001) and aphrodisin in hamsters (Briand et al. 2004). In these species, the identification of the natural endogenous ligands of these proteins was a prerequisite for studying their binding properties and understanding their physiological role.

Porcine olfactory binding proteins have been studied extensively because one of the first mammalian OBP was identified in this species (Dal Monte et al. 1991; Paolini et al. 1998). Besides porcine OBP (pOBP), two other proteins [SAL and Von Ebner's Gland protein (VEG)] were identified and characterized from nasal tissue (Scaloni et al. 2001) and the vomeronasal organ (VNO) (Guiraudie et al. 2003). The natural ligands of SAL were identified as androstenol and androstenone, the two components of the boar sex pheromone (Marchese et al. 1998; Loebel et al. 2000). pOBP extracted from nasal tissue does not bear a ligand (Paolini et al. 1998), contrary to the bovine OBP, which contains 1-octen-3-ol as its natural ligand (Ramoni et al. 2001). Finally, attempts to identify the natural ligand of porcine VEG were unsuccessful (Garibotti et al. 1995; Burova et al. 2000; Scaloni et al. 2001). The human counterpart of porcine VEG, the tear lipocalin, was found to contain fatty acids, cholesterol, triacylglycerol, and fatty alcohols (Glasgow et al. 1995), which were not bound or were only weakly bound by porcine VEG in binding assays (Scaloni et al. 2001).

In a previous study, we used an in-gel binding assay to examine the partitioning of several ligands (fatty acids and steroids) among olfactory binding proteins (OBP, SAL, and VEG) of the respiratory mucosa and the VNO of the pig (Guiraudie et al. 2003). In both tissues, two isoforms of VEG displayed different affinities for the ligands: the slower migrating form (VEG1) bound the steroid progesterone but not fatty acids, whereas the faster migrating form (VEG2) only bound fatty acids. We hypothesized that the natural VEG1 ligand could be a steroid. Here, we report the extraction, derivatization, and characterization by gas chromatography coupled to mass spectrometry (GC-MS) of the natural ligands of porcine VEG1, VEG2, and OBP.

Methods and Materials

Protein Extraction from Biological Samples Animals (Large White pre-pubertal male pigs) were slaughtered in agreement with UE directives about animal welfare. VNO were dissected immediately after death and stored at −80°C (Guiraudie et al. 2003). Native proteins were extracted from 10 mg of VNO by adding 800 µl of a 2:1 solution of chloroform/methanol (v:v) to the tissue in Eppendorf tubes on ice. After a brief vortex homogenization, samples were centrifuged at 13,000×*g* for 10 min at 4°C, to separate the different phases. As the tissues contained water, three phases were obtained: the upper chloroform phase (containing lipids), the water interface containing the tissue, and the methanol phase containing proteins. The methanol phase was dried under vacuum (Speed-Vac, Eppendorf, Le Pecq, France) and stored at −20°C until use.

Binding Assay with Radiolabeled Analogs of Steroids The principle of the in-gel binding assay and its protocol have been fully described (Guiraudie et al. 2003). Tritiated testosterone (17-β-hydroxy-4-androsten-3-one, (1,2,6,7-^{3}H[N])) and progesterone (4-pregen-3,20-dione; (1,2,6,7-^{3}H[N])) were from Sigma-Aldrich (La Verpillière, France) and had a specific activity of 3.7 TBq/mmol and 3.4 TBq/mmol, respectively. An aliquot of VNO extract in sample buffer (identical to Fig. 1) was incubated for 30 min on ice with an ethanolic solution of either tritiated testosterone or tritiated progesterone (5 µl, 3×10^{7} Bq), before analysis by electrophoresis, blotting, and fluorography (Guiraudie et al. 2003).

Identification of the Binding Proteins by In-Gel Digest, Peptide Extraction, and Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) Analysis An aliquot of each biological sample used for ligand extraction was loaded onto a 0.75 mm thick gel, and electrophoresis was run in non-denaturing conditions to check the identity of proteins by peptide mapping (Nagnan-Le Meillour et al. 2009). Peptides resulting from the trypsin digestion were extracted by adding 30 µl of a 0.1% trifluoroacetic acid (TFA) solution in 50% acetonitrile. A solution of α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich) at a concentration of 10 mg/ml in 0.1% 2:1 acetonitrile/TFA (v:v) was used as matrix. The peptides were analyzed on a Voyager DE Pro MALDI-TOF mass spectrometer (Applied Biosystem, Courtaboeuf, France), in positive linear mode. An external calibration of peptides covering the 1,000–4,000 mass range was performed for each measurement. Protein identification was achieved by comparison of measured peptide masses with the theoretical OBP (GenBank accession number NP_998961) and VEG (S77587) peptide maps (http://expasy.org/tools/peptide-mass.html). The SAL was

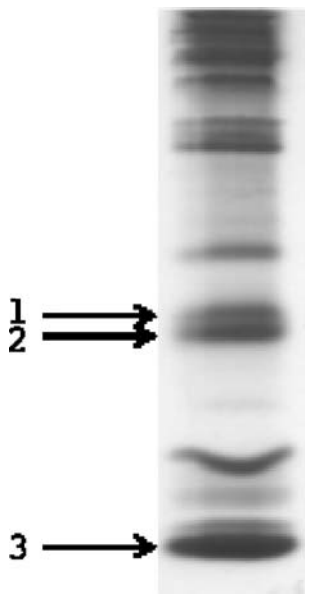

Fig. 1 Separation of pig, *Sus scrofa*, VNO proteins by native-PAGE. Position of bands 1, 2, and 3 used for both peptide mapping and ligand extraction are indicated

Table 1 MALDI-TOF MS analysis of peptides obtained after trypsin digestion of reduced and carboxymethylated bands 1 and 2 from extracts of pig, *Sus scrofa*, vomeronasal organ (VNO) tissue

Modifications	Theoretical mass	Peptide	Measured mass	
			VNO band 1	VNO band 2
PYRR[a]:1	1750.8922	1–16	1750.7416	1750.9074
MSO[b]:22	1751.8672	17–31	1751.7394	1751.9078
	1224.7562	32–42	1224.5975	
	1617.7907	70–83		1617.7398
	1773.8918	70–84	1773.7189	1773.8719
	1489.6958	71–83		1489.6173
	1645.7669	71–84	1645.6264	1645.7855
	918.5658	85–92	918.3688	918.4803
Cys_CAM[c]:101	2323.1023	93–111	2322.8579	2323.1255
	1956.9661	115–131	1956.7757	1956.9635
	2412.2153	115–135		2412.1810
	1986.9403	119–135	1986.7655	1986.9447
Cys_CAM[c]:152	2140.0087	138–157		2140.0189
Sequence coverage (%)			68.15	82.16

[a] PYRR: pyrrolidone carboxylic acid.

[b] MSO: methionine sulfoxide.

[c] Cys_CAM: carbamidomethyl-cysteine.

not included in this study as its endogenous ligands are known.

Extraction of Endogenous Ligands and Derivatization Protein extracts were submitted to electrophoresis in non-denaturing conditions as previously described (Guiraudie et al. 2003). Gels (1.5 mm thickness) were stained with colloidal Coomassie blue (Guiraudie et al. 2003) until the bands just appeared. Placing the gels in water stopped staining, and the band slices were cut immediately with a razor blade and put into an Eppendorf tube. As a control, portions of the gel corresponding to the sample buffer without proteins also were dissected. Band slices were loaded with 500 µl of 2:1 chloroform/methanol (v:v) and ground by using a conical pestle at room temperature. After a brief centrifugation (13,000×*g* for 5 min at room temperature), the supernatant was recovered and dried at room temperature in a rotary evaporator. At room temperature and under harsh extraction, the proteins were denatured, and they liberated their ligands. The remaining water was eliminated in the same conditions as an acetonitrile/water azeotrope obtained after addition of 500 µl acetonitrile dried previously on calcinated calcium chloride (Zanetta et al. 1999).

The dry residue was taken up in 200 µl of dried acetonitrile, and 50 µl of heptafluorobutyric anhydride (HFBAA) (Fluka, Sigma-Aldrich) were added. The samples were submitted to a flash acetylation for 1 min in a sand bath at 150°C. Before GC/MS analyses, individual samples were dried under a stream of nitrogen and supplemented with 100 µl of dried acetonitrile. In order to verify the nature of the fatty ligands, after GC-MS analyses, the samples were evaporated under a stream of nitrogen, then

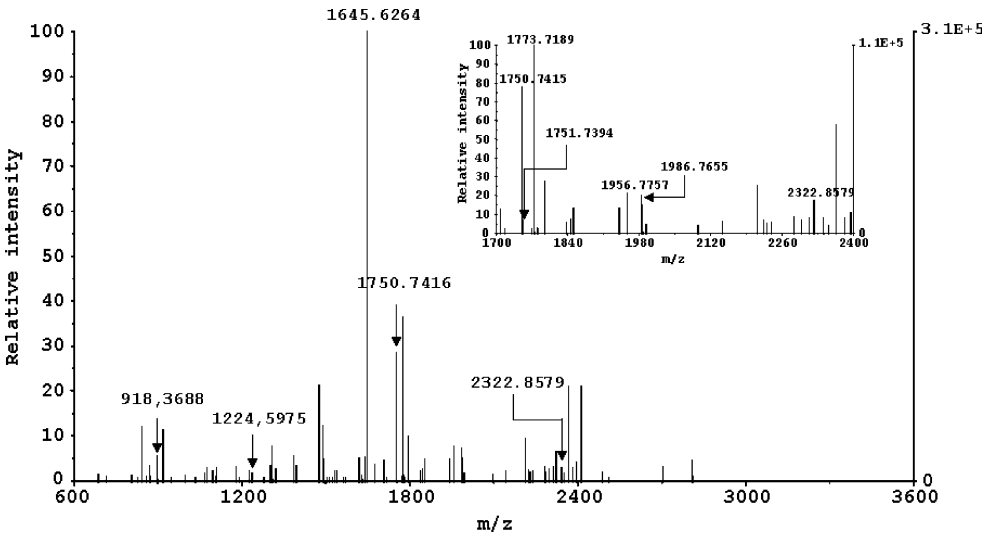

Fig. 2 Monoisotopic mass spectrum of the MALDI-TOF MS analysis of reduced and carboxymethylated band 1 of the pig VNO extract after trypsin digestion. Insert: magnification of the 1,700–2,400*m/z* zone

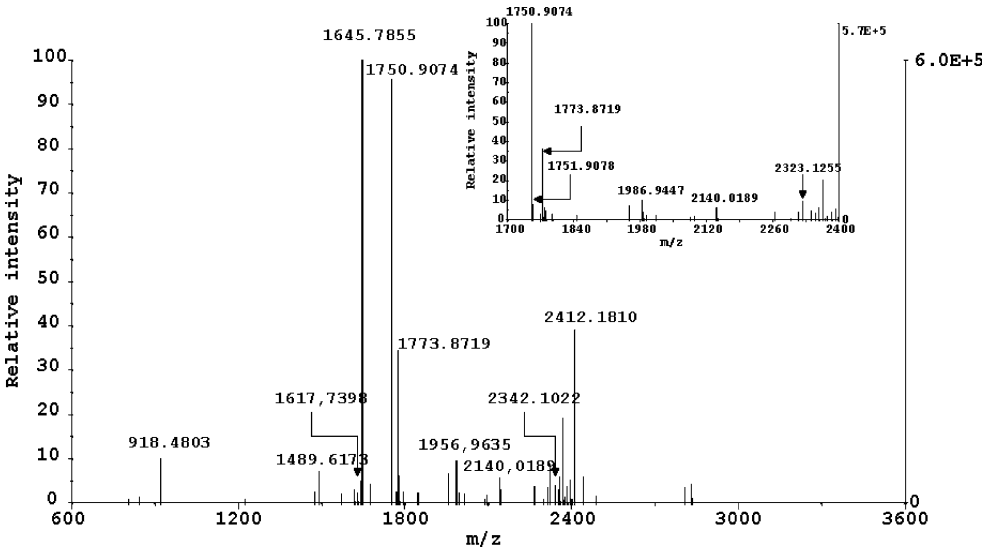

Fig. 3 Monoisotopic mass spectrum of the MALDI-TOF MS analysis of reduced and carboxymethylated band 2 of the pig VNO extract after trypsin digestion. Insert: magnification of the 1,700–2,400*m/z* zone

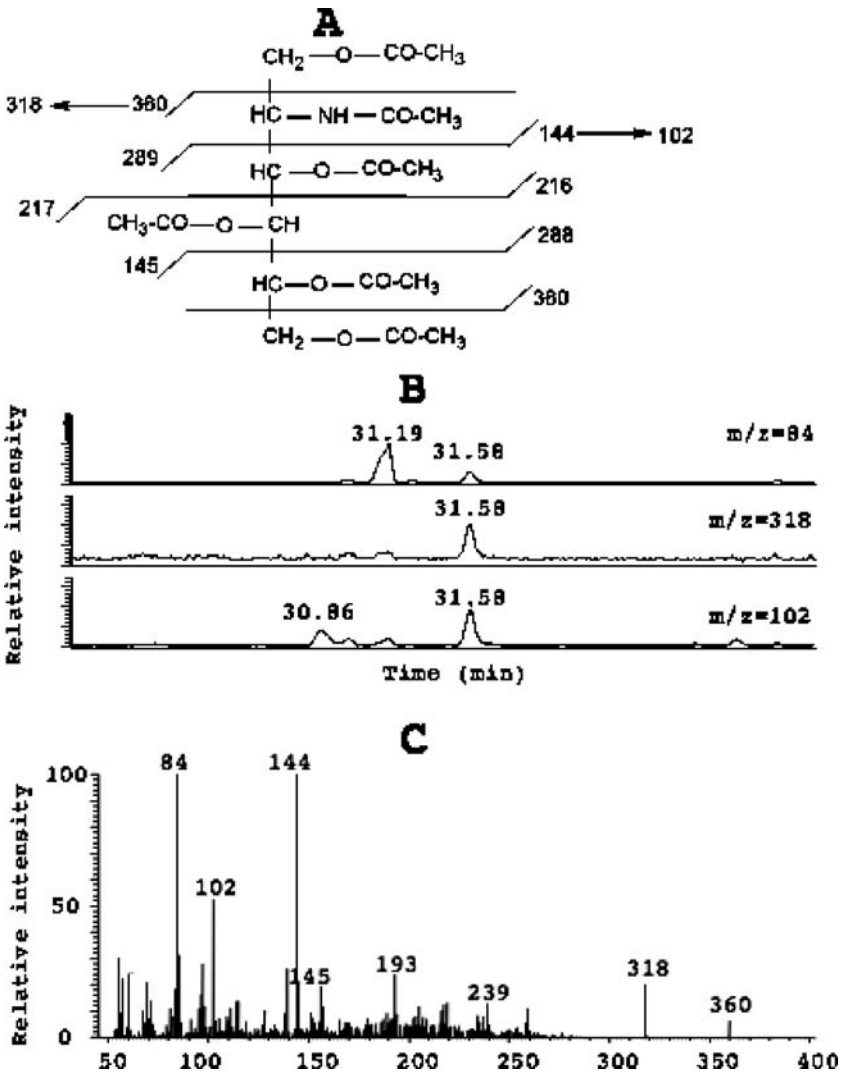

Fig. 4 Characterization of the *O*-GlcNac moiety in pig VEG1. **A)** Fragmentation scheme of peracetylated *O*-GlcNac. **B)** Selected monitoring of ions (*m/z* 84, 102, 318) typical of peracetylated *O*-GlcNac. **C)** Mass spectrum of the peak with R_t = 31.58 min

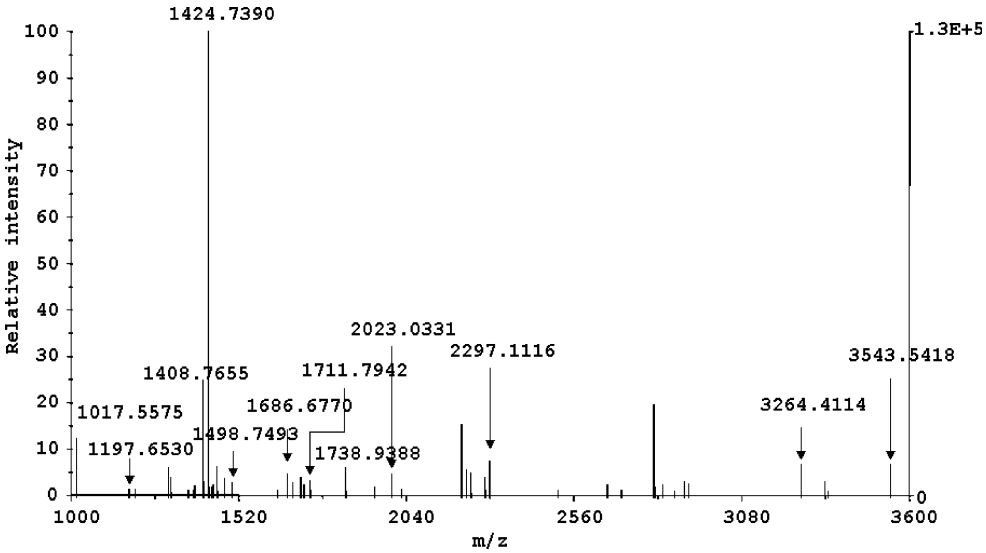

Fig. 5 Monoisotopic mass spectrum of the MALDI-TOF MS analysis of reduced and carboxymethylated band 3 of the pig VNO extract after trypsin digestion

supplemented with 200 µl anhydrous methanol and 200 µl of a diazomethane solution in diethyl ether. The samples were left for 1 h at room temperature, dried under a stream of nitrogen, taken up in dried acetonitrile, and analyzed by GC-MS under the same conditions.

Identification of the Ligands by Gas chromatography Coupled to Mass Spectrometry (GC-MS) Ligands were separated by using a Carlo Erba GC 8000 gas chromatograph equipped with a 25 m×0.32 mm CP-Sil5 CB Low bleed/MS capillary column, 0.25 µm film phase (Chrompack, Les Ulis, France). The temperature of the Ross injector was 260°C, and the samples were analyzed with the following temperature program: 90°C for 3 min, then 5°C/min until 260°C, followed by 20 min at 260°C. The column was coupled to a Finnigan Automass II mass spectrometer (mass detection limit 1000). Analyses were performed routinely in the electron impact (EI) mode (ionization energy 70 eV; source temperature 150°C). In order to preserve the filament of the ionization source, GC/MS records were performed 5 min after the injection of the sample. The EI mode of MS analysis was chosen because it provided fundamental information on the structure of the compound(s) and allowed a general representative total ion current (TIC) response for the different kinds of compounds. Data were recovered between 45 and 1000 *amu.* Integrations of the peaks were performed on the TIC chromatogram with Xcalibur software (Thermo Fisher Scientific, Courtaboeuf, France). The standard compounds (testosterone, fatty acids) were from Sigma-Aldrich.

Characterization of the O-N-acetylglucosamine Moiety The YinOYang server at www.cbs.dtu.dk/services/YinOYang/ (*via* www.expasy.org) was used to predict potential sites of *O*-N-acetylglucosamine (*O*-GlcNac) modification. The presence of *O*-GlcNac was investigated experimentally by using beta-elimination. This reaction occurs when *O*-GlcNac beta-linked serine and threonine residues of proteins are exposed to strongly alkaline conditions (Oda et al. 2001). For beta-

Table 2 MALDI-TOF MS analysis of peptides obtained after trypsin digestion of reduced and carboxymethylated band 3 from extracts of pig, *Sus scrofa*, vomeronasal organ (VNO) tissue

Modifications	Theoretical mass	Peptide	Measured massVNO band 3
PYRR[a]:1	1711.7810	1–15	1711.7942
	1498.7424	16–28	1498.7493
	1408.7041	29–40	1408.7655
MSO[b]:39	1424.6991	29–40	1424.7390
	1197.5633	41–50	1197.6530
	1017.5404	51–58	1017.5575
Cys_CAM[c]:63	3264.4749	59–87	3264.4114
	1686.7354	73–87	1686.6770
MSO[b]:114	3543.8138	88–120	3543.5418
	2022.9978	121–137	2023.0331
	1738.8970	138–152	1738.9388
	2297.0714	138–157	2297.1116
Sequence coverage (%)			100

[a] PYRR: pyrrolidone carboxylic acid.

[b] MSO: methionine sulfoxide.

[c] Cys_CAM: carbamidomethyl-cysteine.

elimination, proteins were separated by native-PAGE as described above, then electroblotted onto PVDF membrane (Immobilon P, Millipore, Saint-Quentin-en-Yvelines, France) as described in Guiraudie et al. (2003). The two bands containing the VEG isoforms were cut with a razor blade and placed immediately (to avoid drying) in a solution of 0.5 M $NaBH_4$, 0.1 M NaOH for 18 h at 45°C. The reaction was stopped by the dropwise addition of acetic acid on ice. After evaporation under nitrogen, methanol was added, then evaporated again to eliminate the methylborates (repeated x 3). The dry sample was resuspended in 1 ml acetic anhydride for 4 h at 100°C to peracetylate the alditols that resulted from the beta-elimination. The peracetylated alditols were extracted with chloroform and analyzed by GC-MS by using a BPX70 capillary column (30 m×0.32 mm) coupled to a Finnigan Automass II mass spectrometer (Thermo Fisher Scientific). The temperature program was from 150°C to 230°C at 3°C/min, then 230°C to 250°C at 5°C/min.

Results

Characterization of Binding Proteins An aliquot of each of the VNO extracts used for ligand identification was loaded onto a 0.75 mm-thick gel in parallel for peptide mapping to

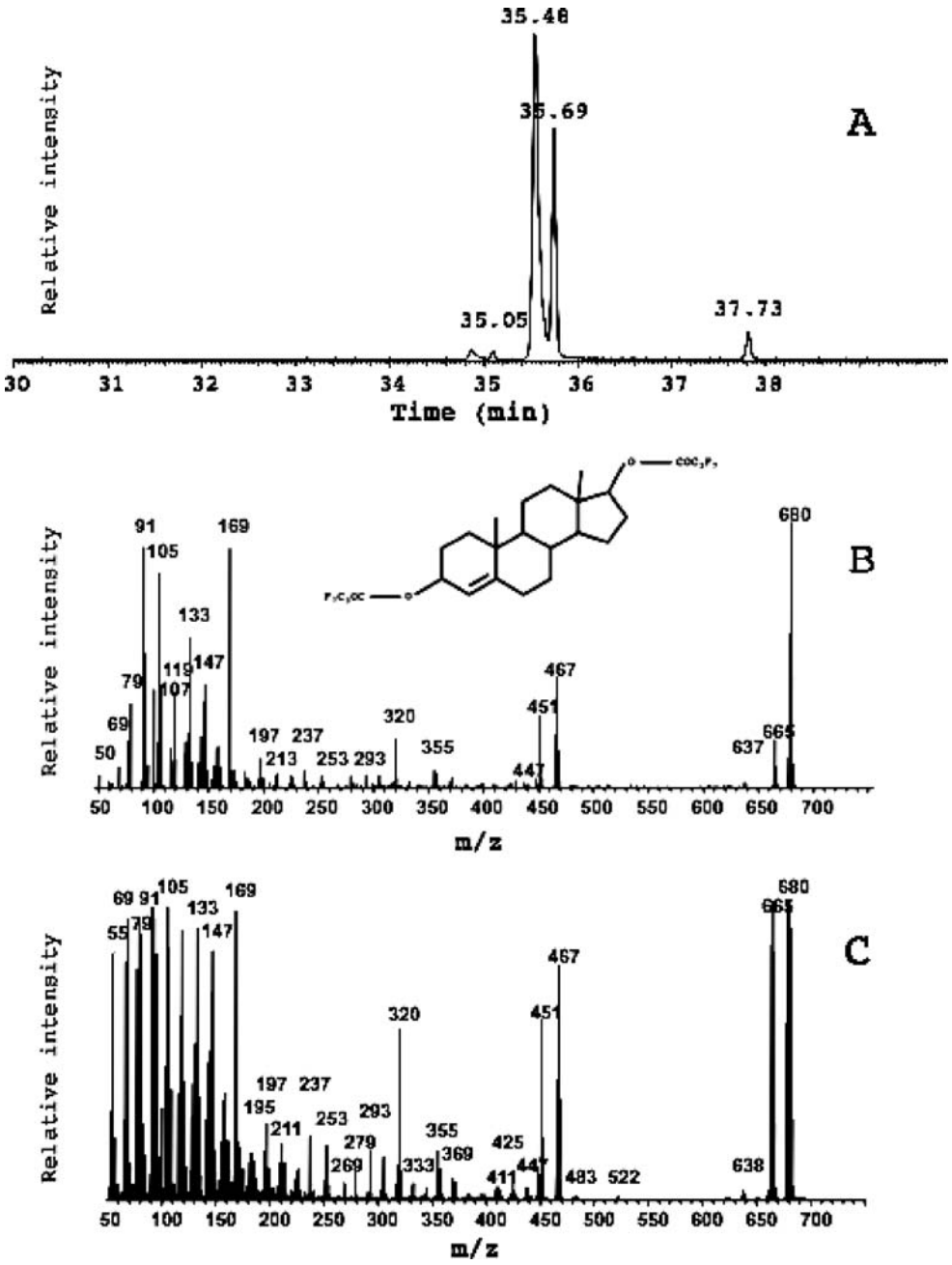

Fig. 6 Characterization of VEG1 ligands. **A)** Total ion chromatogram of ligand extraction from pig VEG1. **B)** Mass spectrum of the peak with R_t = 35.69 min. **C)** Mass spectrum of derivatized standard testosterone (Sigma-Aldrich)

confirm the identity of the proteins used for ligand extraction (Fig. 1). Bands 1 to 3 were analyzed to check the presence of VEG and OBP (Guiraudie et al. 2003). MALDI-TOF MS analysis confirmed the presence of VEG1 in band 1, VEG2 in band 2, and OBP in band 3. A comparison of the peptides from the tryptic digestion of bands 1 and 2 from the VNO to the theoretical peptides from VEG digestion by the same enzyme revealed that the VEG sequence was covered at 68.15% in band 1 and 82.16% in band 2 (Table 1). The difference between the two VEGs is the absence in VEG1 of the peptide 138–157 (*m/z*: 2,140.0087, Figs. 2 and 3) typical of the C-terminus of the protein (Guiraudie et al. 2003). Because the presence of extra-groups linked to peculiar amino acids may explain the absence of peptides (e.g., SAL bears N-glycosylations branched on Asn53, Scaloni et al. 2001), we searched for

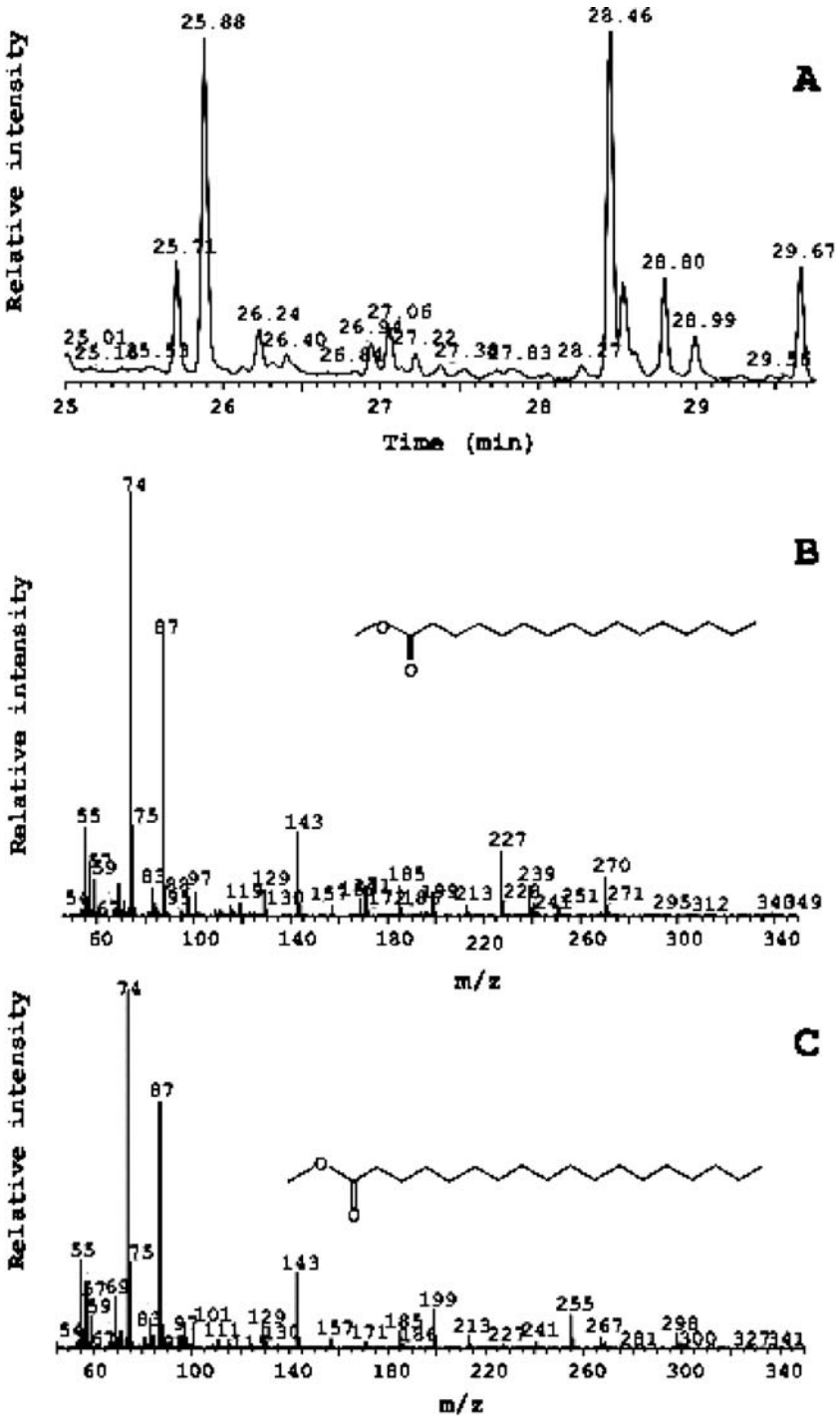

Fig. 7 Characterization of pig VEG2 ligands. **A)** Total ion chromatogram of ligand extraction from VEG2. **B)** Mass spectrum of peak with Rt = 25.71 identified as hexadecanoic acid methyl ester. **C)** Mass spectrum of peak with Rt = 29.67, identified as octadecenoic acid methyl ester. Peaks of Rt = 25.88 and Rt = 28.80 were identified as phthalates, peak of Rt = 28.46 as oleanitrile, all retrieved in the control

potential sites of post-translational modifications in the proteomic database www.expasy.org. The YinOYang server indicated only the possibility of *O*-N-acetylglucosaminylation on Ser149, with a high score (0.67 with a threshold of 0.35). We, thus, performed a beta-elimination on the entire proteins (VEG1 and VEG2) electroblotted onto PVDF, and the presence of glycans was investigated by GC-MS. Ions of *m/z* 84, 102, and 318, typical of the peracetylated *O*-N-acetylglucosamine fragmentation (*O*-GlcNac, Fig. 4A) were searched and retrieved at the retention time of 31.58 min (Fig. 4B) in VEG1 chromatogram. No such ions were found in the VEG2 chromatogram. Standard *O*-GlcNac was injected in the same GC-MS conditions and eluted at a retention time of 31.50 min (data not shown). The mass spectra of the experimental peak of R_t 31.58 and standard *O*-GlcNac were compared, and confirmed the presence of a *O*-GlcNac moiety on the protein (Fig. 4C). Peptide mapping on band 3 of the VNO (Fig. 5) revealed that the peptide sequence of OBP was covered at 100% (Table 2).

Identification of Binding Protein Ligands by GC-MS Bands corresponding to the two isoforms of VEG, and OBP were submitted to ligand extraction, the resulting supernatants were derivatized, and analyzed by GC-MS. The chromatogram of the VEG1 extraction displayed two major peaks of R_t 35.48 and 35.69 (Fig. 6A), whose mass spectra suggested steroids. No data were available for HFB derivatives in the NIST library, but the published analysis

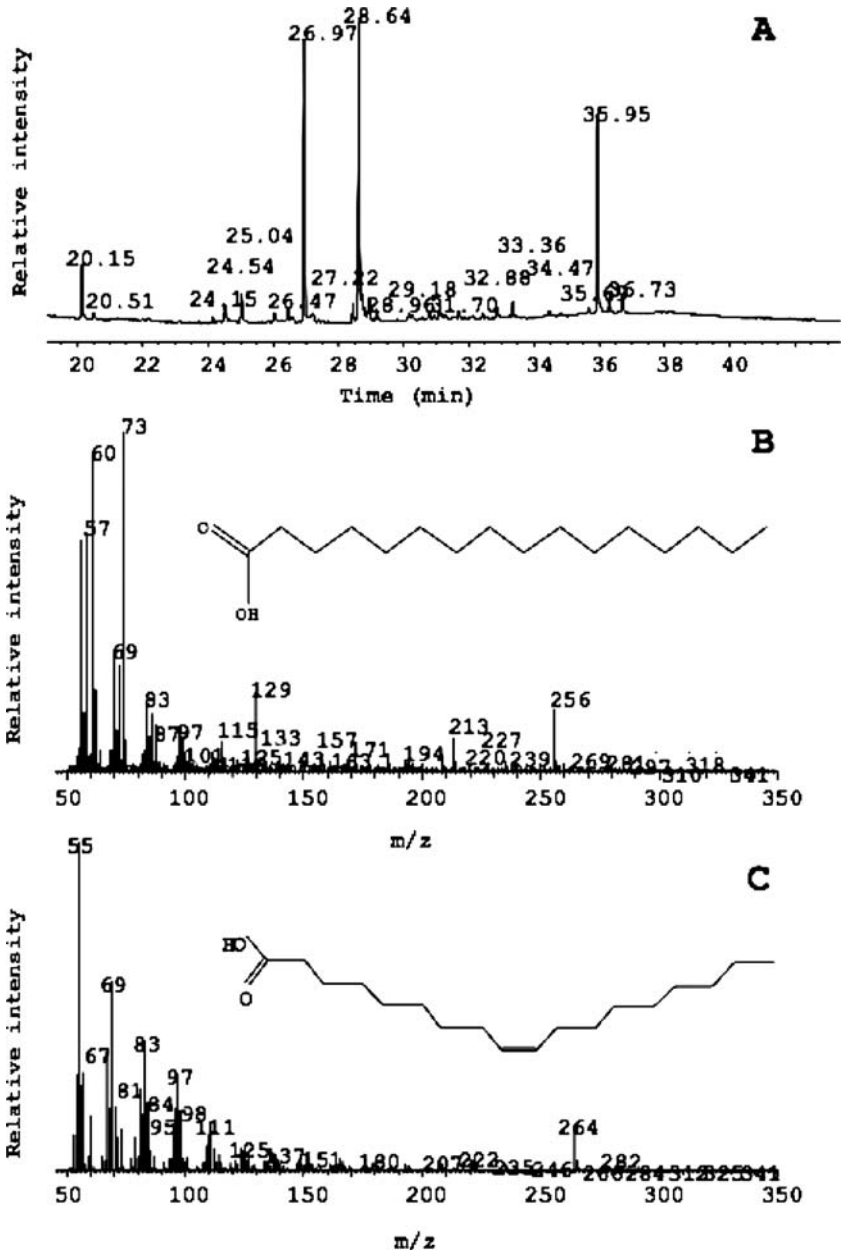

Fig. 8 Characterization of pig OBP ligands. **A)** Total ion chromatogram of ligand extraction from band 3. **B)** Peak with R_t = 26.97 was identified as hexadecanoic acid by comparison with R_t and mass spectrum of the standard. **C)** Peak of R_t = 28.64 was identified as octadecenoic acid. Peaks of R_t = 20.15 min and of R_t = 35.95 corresponded to phthalates

of anabolic steroids of cattle, derivatized with HFBAA (Daeseleire et al. 1998) indicated that the presence of four selected ions (*m/z*: 680, 467, 320, and 355) is typical of testosterone (Fig. 6B). Standard testosterone was derivatized and analyzed under the same conditions. Its mass spectrum (Fig. 6C) was identical to the mass spectrum of the compound with R_t = 35.69 (Fig. 6B). Since the mass spectrum of the compound with R_t = 35.48 was identical, we hypothesize that it corresponds to a testosterone isomer, dihydroxytestosterone, which is secreted together with testosterone, and is the biologically active steroid (see Discussion).

Analysis of the VEG2 ligands (Fig. 7A) showed several peaks of contaminants that also were retrieved in the control sample (oleanitrile and phthalates). We did not detect any steroid. Two fatty acid methyl esters were characterized (match of 100% with the NIST spectra), hexadecanoic acid methyl ester at R_t = 25.71 min (Fig. 7B), and octadecenoic acid methyl ester at R_t = 29.67 min (Fig. 7C).

The chromatogram of OBP ligand extraction showed four major peaks (Fig. 8A). The one at R_t = 26.97 was identified as palmitic acid (hexadecanoic acid, Fig. 8B) and the peak at R_t = 28.64 as oleic acid [(*Z*)-9-octadecenoic acid, Fig. 8C]. Two phthalates were identified at R_t = 20.15 and R_t = 35.95.

Binding Assay with Radiolabeled Analog of Testosterone The results reported above indicated that the chemical structure of ligands bound by VEG1 and VEG2 were different, suggesting a strong specificity in binding properties of the two VEG isoforms. The specific binding of VEG1 to progesterone and of VEG2 for fatty acids had already been demonstrated (Guiraudie et al. 2003). To validate these data, we performed a binding assay with tritiated testosterone, the endogenous ligand of VEG1, and progesterone, as a positive control (Fig. 9). As expected, testosterone was only bound by VEG1, whereas progesterone was strongly bound by VEG1 and weakly bound by VEG2 and OBP.

Discussion

We used HFBAA derivatization to characterize the endogenous ligands of the porcine olfactory binding proteins (VEG isoforms and OBP) because it is a standard method for the GC and/or GC-MS analysis of steroid derivatives (e.g., Daeseleire et al. 1998). Because we did not purify the putative ligands, the samples contained some contaminants coming from electrophoresis sample buffer, principally Tris, sucrose, and also phthalates from Eppendorf tubes (polypropylene), which were easily identified by using EI detection in mass spectrometry (typical 149 ion). These contaminants did not interfere with the identification of the ligands.

We characterized the endogenous ligands of VEG isoforms and OBP from pig VNO, which endogenous ligands were able to displace in a binding assay (Guiraudie et al. 2003; this study). Our analysis showed that VEG2 and OBP ligands are fatty acids or their derivatives, already found in the human counterpart of VEG, the tear lipocalin (Redl 2000). These data also are in accordance with published results obtained from an in-gel binding assay, where VEG2 and OBP bound different fatty acids of the porcine maternal pheromone (Guiraudie et al. 2003). We also have demonstrated that the steroid progesterone was bound only by VEG1, suggesting a strong binding specificity for steroids. Indeed, we have identified the previously unknown natural ligand of VEG1 as testosterone, differing from progesterone by the substitution of an alcohol instead of a methyl-ketone on carbon 17. Testosterone is the major compound secreted by the testes and is metabolized into dihydrotestosterone, which is transported *via* the blood to the submaxillary gland (Babol et al. 1996) by binding to various proteins (sex hormone binding globulin and in a lesser extent, albumin). The expression of VEG is associated with glands and organs involved in sexual communication. Its high concentration in saliva, together with androstenone and androstenol, suggests a role in mating behavior of pigs that could be investigated by behavioral assays. In this context, VEG1 of the VNO has to be considered as a pheromone-binding protein [as is the case with SAL (Marchese et al. 1998; Loebel et al. 2000)]. The presence in the same compartment of both components involved in sexual communication and OBP has been documented in insects. In *Drosophila*, the fatty acid derivative *cis*-vaccenyl acetate is transferred from the male to the female during copulation bound to a CSP (Jallon et al. 1981; GenBank accession number U08281). In the Lepidoptera, CSPs are expressed in the pheromonal gland, at least to solubilize pheromone components, which are fatty acid derivatives (Jacquin-Joly et al. 2001).

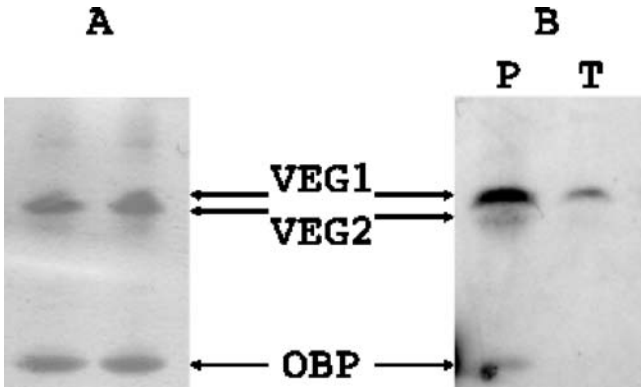

Fig. 9 In-gel binding assay between pig VNO extract and tritiated steroids. **A)** PVDF membrane stained with Ponceau red after electroblotting of VNO proteins. Position of VEG1, VEG2 and OBP is indicated. **B)** Fluorography after a 1-wk exposure, indicating the location of the radiolabeled progesterone (P) and testosterone (T)

As the only difference that we identified between VEG1 and VEG2 is the *O*-GlcNac moiety on VEG1, we assume that the binding specificity of VEG1 to steroids is linked to the presence of the *O*-GlcNac group on the protein. This result is unusual: indeed, the *O*-linked N-acetylglucosaminylation of proteins is described as a nuclear and cytoplasmic modification (Torres and Hart 1984) and is not supposed to occur on secretory proteins, i.e., proteins synthesized *via* the endoplasmic reticulum, that bears a signal peptide as olfactory binding proteins. *O*-N-acetylglucosaminylation and phosphorylation can occur on the same Ser and Thr residues in a reversible mechanism called YinOYang (Corner and Hart 2001). The enzyme responsible for the *O*-GlcNac linkage (*O*-GlcNac transferase, OGT) was not retrieved in the reticulum compartment (Hart et al. 2007). Although there is evidence for the phosphorylation of extracellular proteins by ectokinases (e.g., Nath et al. 2008), to date no protein localized in the extracellular pathway has been described to bear *O*-GlcNAc residues. However, the linkage of *O*-GlcNac to an olfactory binding protein was already reported for hamster aphrodisin (Briand et al. 2000). This surprising result, however, was not discussed by these authors, despite the fact that two potential *O*-GlcNac sites (S58, T148) are predicted by the YinOYang server (SwissProt accession number Q9Z1I7). This unusual post-translational modification for secretory proteins could be a special feature of Bowman's gland secreting cells and the study of VEG1 biosynthesis pathway in such cells is currently ongoing.

Until now, the impact of post-translational modifications on the binding properties of OBPs (*sensu largo*) has not been fully investigated. The *O*-GlcNac modification creates several isoforms of OBPs, which possess different and specific binding affinities for pheromone components. The existence of such variants extends the possibility of coding for odorants by OBPs, and it suggests that the binding of odorants by OBPs is an initial discrimination step, preceding binding to olfactory receptors. This hypothesis is novel for the field of Mammalian olfaction where OBPs have been considered mainly as passive carriers of odorant molecules.

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Phosphorylation of Native Porcine Olfactory Binding Proteins

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Abstract The identification of various isoforms of olfactory binding proteins is of major importance to elucidate their involvement in detection of pheromones and other odors. Here, we report the characterization of the phosphorylation of OBP (odorant binding protein) and Von Ebner's gland protein (VEG) from the pig, *Sus scrofa*. After labeling with specific antibodies raised against the three types of phosphorylation (Ser, Thr, Tyr), the phosphate-modified residues were mapped by using the beta-elimination followed by Michael addition of dithiothreitol (BEMAD) method. Eleven phosphorylation sites were localized in the pOBP sequence and nine sites in the VEG sequence. OBPs are secreted by Bowman's gland cells in the extracellular mucus lining the nasal cavity. After tracking the secretion pathway in the rough endoplasmic reticulum of these cells, we hypothesize that these proteins may be phosphorylated by ectokinases that remain to be characterized. The existence of such a regulatory mechanism theoretically increases the number of OBP variants, and it suggests a more specific role for OBPs in odorant coding than the one of odorant solubilizer and transporter.

Keywords BEMAD · MALDI-TOF mass spectrometry · Olfaction · Phosphorylation · Polyacrylamide gel electrophoresis · Porcine odorant binding protein · Secretory protein · Von Ebner's gland protein · Western blot

Chrystelle Le Danvic and Fanny Brimau contributed equally to the work.

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Introduction

Despite extensive studies of their structure and binding properties, the role of odorant binding proteins (OBPs) in the detection of odorant molecules still is not completely understood (Tegoni et al. 2000). These small soluble proteins are secreted in the mucus that lines the nasal cavity in mammals where olfactory receptor neurons are located. One major unresolved question about OBPs is their interaction with olfactory receptors, and the nature of the olfactory receptor ligands. Two major hypotheses have been proposed: 1) The ligand is the odorant molecule itself, solubilized and transported to the receptor by OBPs. In this scheme, the binding between odorant molecules and OBPs is unspecific, and this implication is supported by the low number of OBPs found in the mucosa of each animal species, and by their broad spectrum of binding of

hydrophobic molecules (Dal Monte et al. 1993; Herént et al. 1995). OBPs also are assumed to concentrate odorants and/or to scavenge them from receptors in a deactivation process (Pelosi 2001). 2) The ligand is the complex formed by the specific binding between a given odorant molecule and a specific OBP. This hypothesis involves a conformational change of the OBP upon ligand binding, which confers an "activated" form to the complex, able to interact with a specific receptor. It also implies that the number of OBPs should be higher than yet described. The micro diversity of OBPs has not been fully investigated. However, Paolini et al. (1998) reported that the porcine OBP is the major OBP of nasal mucosa, although perhaps not the only one. Microheterogeneity of OBPs related to primary structure differences has also been reported in porcupine (Ganni et al. 1997).

We have used a ligand-oriented approach to identify the proteins involved in the specific binding of pheromone components in the nasal mucosa of pre-pubertal pigs, *Sus scrofa* (Guiraudie et al. 2003). Three OBPs were identified as Salivary Lipocalin (SAL), Von Ebner's gland protein (VEG), and porcine odorant binding protein (pOBP), by immunodetection with specific antibodies, peptide mapping, and cloning of the encoding cDNA. The binding assay, performed in non-denaturing conditions, revealed two isoforms of VEG, with different binding properties (Guiraudie et al. 2003). We have shown recently that the binding specificity of VEG1 isoform for steroids is governed by the presence of an *O*-GlcNac moiety on the protein (Le Danvic et al. 2009). Such a specificity of ligand binding, linked to OBPs isoforms, strongly favors the hypothesis that specific complexes could exist between odorants and OBPs. Furthermore, we observed that the purification of olfactory binding proteins by anion exchange HPLC provided several isoforms eluted at different NaCl concentrations, suggesting different charges coming from post-translational modifications (unpublished results). Here, we report the identification, characterization, and localization of post-translational modifications of porcine OBP and VEG.

Methods and Materials

Protein Extraction from Respiratory Mucosa Animals (Large White adult sows of *Sus scrofa*) were slaughtered in agreement with UE directives about animal welfare. Respiratory mucosa (RM) was dissected immediately after death and stored at −80°C. Native proteins were extracted from 10 mg of RM with 800 µl of 2:1 chloroform/methanol (v:v, Sigma-Aldrich, La Verpillière, France) on ice. After a brief homogenization by vortexing, the samples were centrifuged at 12,100×*g* for 10 min at 4°C. The methanol phase was dried under vacuum (Speed-Vac, Eppendorf, Le Pecq, France) and stored at −20°C until use.

Dephosphorylation Enzymatic Treatment RIPA buffer (150 mM NaCl, 1% NP40, 0.5 mM DTT in 50 mM Tris/HCl pH=8.5) was added to protein samples and incubated 16 h at 37°C with 2 units of alkaline phosphatase (rAPid alkaline phosphatase, Roche Diagnostics, Meylan, France) for 10 µg of total protein.

Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting Samples were analyzed by 16.8% PAGE gels in denaturing and reducing conditions (SDS-PAGE) as previously described (Guiraudie et al. 2003; Nagnan-Le Meillour et al. 2009). Gel staining (overnight in Coomassie blue R solution) and Western blotting were performed as described in Guiraudie et al. (2003), except that for the Western blot, membranes were saturated with 5% non-fatty milk before incubation with anti-pOBP and -VEG antibodies, or with 3% bovine serum albumin (BSA, Sigma-Aldrich) for anti-phosphorylation antibodies. Rabbit polyclonal antibodies anti-phosphoserine, anti-phosphothreonine, and anti-phosphotyrosine were used at 1:500, 1:500, and 1:2,000 dilutions, respectively, following recommendations of the manufacturer (Zymed laboratories, Invitrogen, Cergy-Pontoise, France). Rabbit polyclonal antibodies were raised in our laboratory against recombinant purified pOBP (Nagnan-Le Meillour et al. 2009). They were used at a 1/20,000 dilution, and rabbit polyclonal anti-VEG antibodies at a 1/10,000 dilution (provided by Professor Paolo Pelosi). Membranes then were incubated with secondary antibodies at a 1:50,000 dilution (anti-rabbit horseradish peroxidase-labeled antibodies, Amersham Biosciences, GE Healthcare, Templemars, France). Detection was carried out with ECL Plus Western Blotting Detection Kit (Amersham Biosciences).

Characterization of Native Proteins by In-gel Digest, Peptide Extraction and MALDI-TOF MS Analysis Band slices containing pOBP and VEG were treated as previously described (Nagnan-Le Meillour et al. 2009), with Trypsin Gold (Mass Spectrometry grade, Promega, Charbonnières, France, 10 ng/µl). Peptides resulting from the trypsin digestion were extracted with two incubations in 45% acetonitrile in 10% formic acid at 30°C for 15 min, followed by an incubation in 95% acetonitrile in 5% formic acid at room temperature (RT) for 15 min. The extracts were dried under vacuum and solubilized in 5 µl of 0.1% trifluoroacetic acid (TFA) before desalting and concentration on a C18 column (Vivapure C18, microspin columns, Sartorius, Palaiseau, France). Peptides were eluted with different percentages of acetonitrile (12.5, 25, 50, and 80%) directly into the matrix (α-cyano-4-hydroxycinnamic acid, Sigma-Aldrich) prepared at a concentration of 10 mg/ml in

50% acetonitrile, 0.1% TFA, and were analyzed on a Voyager DE Pro MALDI-TOF mass spectrometer (Applied Biosystems, Courtaboeuf, France). The instrument was used in reflector mode, measuring peptide masses in a range of 500 to 4,000 Da. Calibration points were based on the masses of the matrix cluster or trypsin autolysis products (*m/z* 842, 2211). Protein identification was performed by comparison of measured peptide masses with the theoretical porcine OBP (GenBank accession number NP_998961) and VEG (S77587) peptide maps (http://expasy.org/tools/peptide-mass.html).

Mapping Phosphorylation Sites in Proteins As a first approach, the NetPhos server at http://www.cbs.dtu.dk/services/NetPhos/ (Blom et al. 1999) was used to predict potential phosphorylation sites in pOBP and VEG sequences. The sites of phosphorylation were experimentally determined by using a method involving mild beta-elimination followed by Michael addition of dithiothreitol (BEMAD) adapted from Wells et al. (2002). Proteins were extracted and treated as they were for peptide mapping (above), except that they were not reduced and alkylated before treatment with trypsin, to avoid the addition of DTT on cysteines. Peptides resulting from trypsin digestion were suspended in 50 mM Tris/HCl containing 1 mM $MgCl_2$ and alkaline phosphatase (1 U/10 µl). The reaction was incubated overnight at 37°C, then dried in a speed-vac. Dried peptides were β-eliminated and subjected to Michael addition with DTT by resuspension in 200 µl of BEMAD solution (1% triethylamine, 0.1% NaOH, 20% ethanol, 10 mM DTT) and incubated at 50°C for 2.5 h. The reaction was stopped by addition of TFA to a 1% final concentration. Peptides were cleaned up via reverse-phase Vivapure C-18 micro columns (Sartorius) with different acetonitrile percentages in 0.1% TFA (12.5, 25, 50, and 80%) and dried. DTT modified peptides were enriched by thiol affinity chromatography according to Whelan and Hart (2006). Thiopropyl Sepharose 6B resin (Amersham Biosciences) was swelled and washed in column buffer containing 150 mM NaCl and 1 mM EDTA in 20 mM Tris/HCl, pH 7.6 (TBS/EDTA). The resin was transferred into a Vivaclear Mini Clarifying filter (Sartorius) and washed with 7 ml of TBS/EDTA by centrifugation. Peptides were resuspended in 200 µl TBS/EDTA and loaded onto the thiol column for 4 h at RT by rotation. The column then was washed with 15 ml of TBS/EDTA, and peptides were eluted by three washes of 150 µl TBS/EDTA containing 20 mM of free DTT. They were acidified by 0.1% TFA and cleaned up with reverse phase Vivapure C-18 Micro spin columns (Sartorius). Peptides were eluted directly into the matrix (10 mg/ml of α-cyano-4-hydroxycinnamic acid in 0.1% TFA, 50% acetonitrile) and analyzed by MALDI-TOF MS as above.

Results and Discussion

Heterogeneity of Porcine OBP and VEG The respiratory mucosa (RM) extracts were submitted to electrophoresis in native (Fig. 1A) or denaturing conditions (Fig. 1B), and the respective positions of pOBP and VEG were determined by peptide mapping. Both spectra (Fig. 2) and reconstituted maps (Table 1) indicated that the two proteins co-migrate in the same band in denaturing conditions (Fig. 1B). This result is in accordance with the calculated molecular weights of the proteins (17,935 Da for pOBP and 17,441 Da for VEG) and with previous data obtained by using the same electrophoresis conditions for pOBP (Nagnan-Le Meillour et al. 2009). Each peptide could be assigned unambiguously to one or the other protein, as none of them was found to be common to pOBP and VEG (Table 1). After electrophoresis in native conditions, pOBP and VEG displayed reproducible heterogeneity (Fig. 1A), which was not detected under reducing and denaturing conditions of SDS-PAGE (Fig. 1B). These different forms can result from different global charges, due to post-translational modifications, such as phosphorylation on Ser, Thr, and Tyr residues. The NetPhos server (www.cbs.dtu.dk/services/NetPhos/) indicated that several sites in the primary structure could potentially be phosphorylated: Ser13, Ser24, Ser41, Ser67, Tyr78, and Tyr82 in pOBP, and Ser24, Ser35, Ser149, Thr23, and Tyr97, in VEG. We, thus, performed Western blotting with commercial specific antibodies, raised against the three types of phosphorylation, to test the hypothesis that both proteins are phosphorylated.

Immunodetection of Phosphorylations with Specific Antibodies Preliminary experiments were run under native conditions, where the diversity of binding proteins is the

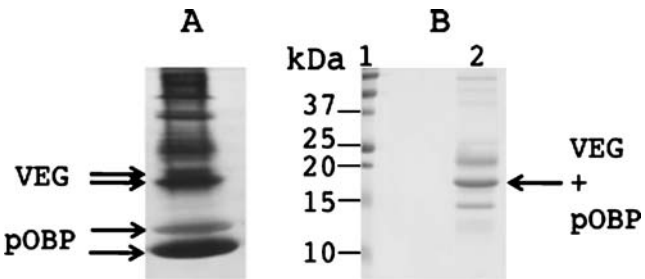
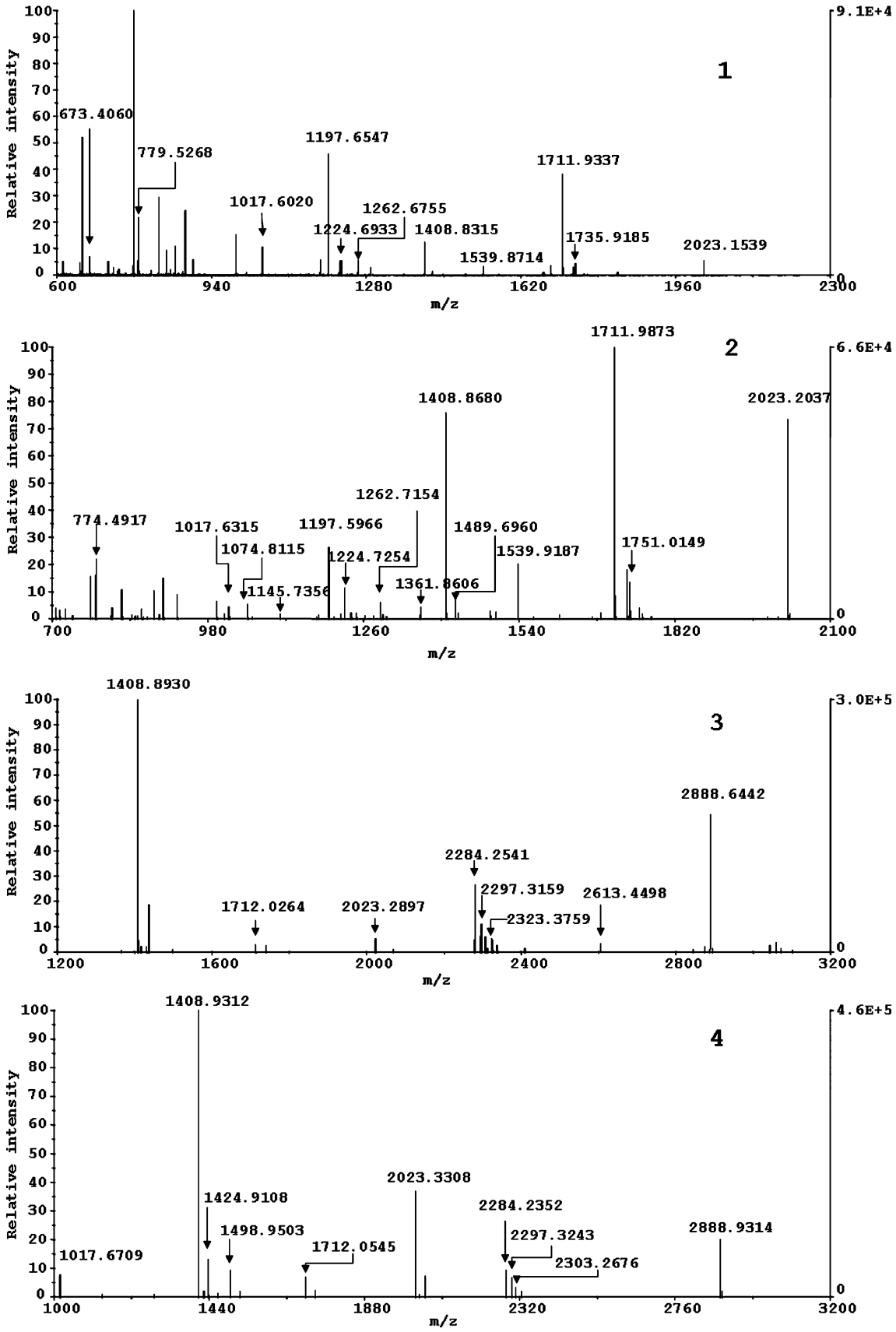


Fig. 1 Separation of protein extracts of pig, *Sus scrofa*, respiratory mucosa (RM) tissue: **A** Native-PAGE and **B** SDS-PAGE. 1: Molecular weight markers in kilodaltons (KDa, Precision plus Protein All Blue, Bio-Rad), 2: RM extract. Coomassie blue staining (16.8% acrylamide). The position of VEG and pOBP, deduced from the peptide mapping analysis, is indicated by the arrow. Note that the quantity of sample in native-PAGE is 20-fold higher than that loaded in the SDS-PAGE gel (same quantity as used in the Western blot experiments after SDS-PAGE)


1
Relative intensity
m/z
673.4060
779.5268
1197.6547
1711.9337
1017.6020
1262.6755
1224.6933
1408.8315
1735.9185
2023.1539
1539.8714
9.1E+4
2
1711.9873
1408.8680
2023.2037
1262.7154
774.4917
1017.6315
1197.5966
1489.6960
1074.8115
1539.9187
1751.0149
1224.7254
1361.8606
1145.7356
6.6E+4
3
1408.8930
2888.6442
2284.2541
2297.3159
2613.4498
1712.0264
2023.2897
2323.3759
3.0E+5
4
1408.9312
2023.3308
1424.9108
2284.2352
1498.9503
2297.3243
2888.9314
1712.0545
1017.6709
2303.2676
4.6E+5

◀ **Fig. 2** Monoisotopic mass spectra from MALDI-TOF MS analysis of reduced and carboxymethylated band slice containing pOBP and VEG (SDS-PAGE) of the pig, *Sus scrofa*, respiratory mucosa (RM) extract after trypsin digestion (untreated sample). Peptides were eluted with different percentages of acetonitrile: 12.5% (**1**), 25% (**2**), 50% (**3**), and 80% (**4**)

most detectable. Unfortunately, phosphate groups often are buried into the folded protein and unreachable by antibodies. As expected, under these conditions, the Western blot with the three antibodies was negative. A Western blot then was performed after SDS-PAGE, and the band containing the two co-migrating proteins (Fig. 3A) was labeled by antiphosphoserine (Fig. 3B), antiphosphotyrosine (Fig. 3C), but not by antiphosphothreonine, which is consistent with the prediction of the NetPhos server of only one potential phosphorylation site on VEG Thr23. To verify the accuracy of the labeling, half of the RM sample was treated with alkaline phosphatase (AP) to remove any phosphate group, and both treated and untreated samples were submitted to Western blot analysis with the three specific antibodies. With antiphosphoserine antibodies, we observed a significant reduction of the signal after AP treatment (Fig. 4A), unless a total knock out. We obtained the same results with antiphosphotyrosine antibodies (data not shown). In both cases, we checked for the presence of pOBP and VEG after treatment by Western blot with anti-pOBP and anti-VEG antibodies (Fig. 4B and C, respective-

Table 1 MALDI-TOF MS analysis of peptides obtained after trypsin digestion of a reduced and carboxymethylated band slice containing pOBP and VEG from extracts of pig, *Sus scrofa*, respiratory mucosa (RM) tissue

Modifications	Theoretical mass (Da)$[M + H]^+$	Peptide	Measured mass $[M + H]^+$	
			Untreated	Treated with AP
		pOBP		
PYRR[a]:1	1711.7809	1–15	1711.9337 (1[b])	1711.8192 (2)
	1498.7424	16–28	1498.9503 (4)	1498.8287 (3)
	2888.4287	16–40	2888.6442 (3)	
	1408.7041	29–40	1408.8315 (1)	1408.7765 (1)
	1197.5633	41–50	1197.5966 (2)	1197.7011 (2)
	1361.7099	48–58	1361.8606 (2)	1361.7923 (2)
	1017.5404	51–58	1017.6315 (2)	1017.5799 (2)
	1539.7359	59–72	1539.9187 (2)	1539.9068 (2)
	2613.2930	88–111	2613.4498 (3)	2613.4177 (3)
	1262.5746	121–131	1262.7154 (2)	
	1537.7380	121–133		1537.7380 (3)
	2022.9978	121–137	2023.1539 (1)	2023.1215 (2)
	779.4410	132–137	779.5268 (1)	
	2240.0499	138–157		2240.3284 (3)
Cys_CAM[c]:155	2297.0714	138–157	2297.3159 (3)	
		VEG		
PYRR[a]:1	1750.9122	1–16	1751.0149 (2)	1750.9928 (2)
	1735.8723	17–31	1735.9185 (1)	1735.9633 (2)
	1145.5507	21–31		1145.6059 (3)
	1224.7562	32–42	1224.6933 (1)	1224.9781 (1)
	1617.7907	70–83		1617.9495 (2)
	1489.6958	71–83	1489.6960 (2)	
	1074.6670	84–92	1074.8115 (2)	1074.7407 (2)
	1145.7292	85–94	1145.7356 (2)	1145.7576 (2)
	2266.0808	93–111		2266.1321 (2)
Cys_CAM[c]:101	2323.1023	93–111	2323.3759 (3)	
	774.4654	112–118	774.4917 (2)	774.5959 (2)
MSO[d]:112	2303.1336	112–131	2303.2676 (4)	
	1986.9403	119–135		1987.3511 (2)
	673.3991	132–137	673.4060 (1)	
	2284.1350	136–157	2284.2541 (3)	2284.3648 (3)

[a] PYRR: pyrrolidone carboxylic acid

[b] Peptides were eluted and recovered at different percentages of acetonitrile: (1) 12.5%, (2) 25%, (3) 50%, (4) 80% (cf. spectra in Fig. 2)

[c] Cys_CAM: carbamidomethylcysteine

[d] MSO: methionine sulfoxide

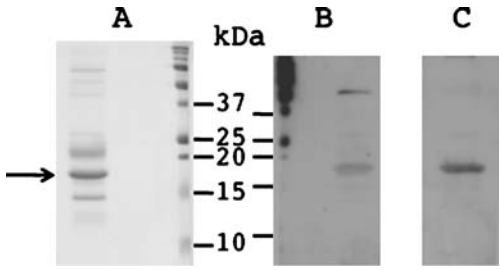


Fig. 3 Immunodetection of phosphorylated proteins in respiratory mucosa (RM) samples of the pig, *Sus scrofa*, by Western blot and ECL Plus detection. **A** Coomassie blue staining of the same sample as used for Western blot analysis. Arrow indicates the band containing pOBP and VEG. **B** antiphosphoserine antibodies (1:500 dilution, 2 min exposure). **C** antiphosphotyrosine antibodies (1:2,000 dilution, 2 min exposure). Discrepancy between relative positions of molecular weight markers in A and B/C comes from the swelling of the gel (**A**) in water

ly). The corresponding band was cut from a SDS-PAGE gel run under the same conditions, and it was submitted to peptide mapping. The use of different percentages of acetonitrile allowed elution of most of the expected peptides. Indeed, the VEG sequence was recovered at 84.1%, and the pOBP one at 84.7% with or without AP treatment, indicating that the proteins were not degraded by the dephosphorylation (Table 1). These data indicated that one of the two proteins, or both, are phosphorylated.

Mapping of Phosphorylation Sites in pOBP and VEG Experiments described above did not permit us to conclude whether one or both proteins are phosphorylated. Thus, the sites were located in proteins experimentally by the BEMAD method followed by MALDI-TOF MS analysis. In MALDI-TOF MS, the *O*-linked phosphate groups are labile and decayed by the laser during desorption of peptides from the matrix (Mann et al. 2002). The use of beta-elimination of phosphate from Ser, Thr, and Tyr residues by alkaline phosphatase, followed by the attack of the nucleophile DTT confers a tag of defined molecular mass (136.2 Da) to the peptides bearing phosphate groups, that are subsequently enriched by the use of thiol columns. The band slice containing pOBP and VEG was submitted to enzymatic digestion and BEMAD modification. The measured masses of resulting peptides were compared to a theoretical list of potential DTT-modified peptides calculated from the pOBP and VEG protein sequences (Table 2). Two different enzymes (Trypsin-T or Chymotrypsin-CT or both) were used for the site analysis. When using either T or CT, many ambiguous sites remained, and it was necessary to perform T and CT treatments successively to obtain the pOBP and VEG phosphorylation sites. Mass spectral analysis of peptides eluted with 50% acetonitrile after T + CT treatment, BEMAD and thiol column enrichment, revealed that in addition to DTT-modified peptides (Table 2), some unmodified peptides, typical of the pOBP and VEG digestion were present (Fig. 5). Five peptides resulted from the pOBP digestion with T + CT (mass 655.3903 corresponding to peptide 51–55), or T (mass 2224.0593, peptide 134–152; mass 2239.8924, peptide 138–157, and mass 2296.9473, peptide 138–157 with Cys_CAM:155) and VEG digestion with T (mass 2265.9512, peptide 93–111). The ratio between DTT-modified and unmodified peaks could not be determined, as the modified peptides were enriched by the use of thiol columns. We suggest that phosphorylated peptides are present in small quantities in the RM extracts, as the unmodified peaks are much more abundant than modified ones, even after enrichment (e.g., mass 2224.0593). The other DTT-modified peptides (Table 2), were obtained with other acetonitrile percentages (12.5, 25, and 80%) and different enzymatic treatment (T or CT). The corresponding spectra are provided in Supplementary data.

In pOBP, the phosphorylation of the following residues was predicted by the NetPhos software: Ser13, Ser24, Ser41, Ser 67, and Tyr82 (Ser24, Ser149, Tyr97, and Thr23 in VEG), whereas Ser23, Ser49, Ser57, Tyr52, Thr71, and Thr122 were not predicted (Ser91, Ser153, Thr54, Thr151, and Tyr87 in VEG). The occurrence of masses indicating the phosphorylation of one or more residues in the same

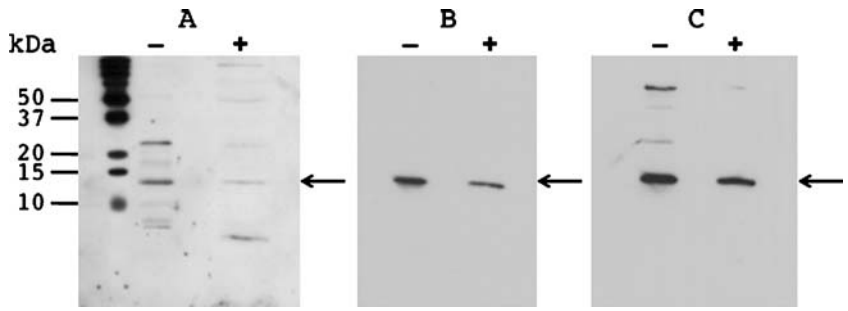


Fig. 4 Analysis of alkaline phosphatase action by Western blot and ECL Plus detection. **A** antiphosphoserine antibodies (1:500 dilution, 2 min exposure). **B** anti-pOBP antibodies (1:20,000 dilution, 15 sec exposure). **C** anti-VEG antibodies (1:10,000 dilution, 15 sec exposure). (−) untreated sample, (+) treated sample, kDa molecular weight markers. Arrows indicate the band containing VEG and pOBP in each experiment

Table 2 BEMAD performed on SDS-PAGE band containing pOBP and VEG from pig, *Sus scrofa*, respiratory mucosa tissue, identifies the sites of phosphorylation

Calculated mass (Da)			Measured mass [+]	Peptide	Peptide sequence
No DTT	1 DTT	2 DTT			
			pOBP		
533.2929	669.4929		669.3701 (T + CT; 50%)	11–15	ELS(*)GK
1312.6691	1448.8631		1448.7923 (T + CT; 50%)	17–28	ITSY(°)IGS(°)S(°)DLEK
848.4360		1120.8360	1120.6106 (T + CT; 25%)	21–28	IGS(*)S(*)DLEK
1950.9807	2087.1807		2086.9766 (CT; 25%)	21–38	IGS(°)S(°)DLEKIGENAPFQVF
1950.9807		2223.3807	2223.2450 (CT; 50%)	21–38	IGS(*)S(*)DLEKIGENAPFQVF
1140.5354	1276.7354		1276.6478 (T + CT; 50%)	39–47	MRS(*)IEFDDK
1197.5633	1333.7633		1333.6166 (T; 50%)	41–50	S(°)IEFDDKES(°)K
1197.5633		1469.9633	1469.8707 (T; 25%)	41–50	S(*)IEFDDKES(*)K
1357.6634		1630.0634	1630.4646 (CT; 25%)	45–55	DDKES(*)KVY(*)LNF
1302.5670	1438.7670		1438.5673 (CT; 50%)	56–66	FS(*)KENGICEEF
1539.7359	1675.9359		1675.9154 (T; 25%)	59–72	ENGICEEFS(°)LIGT(°)K
994.4588	1130.6588		1130.6098 (T + CT; 25%)	79–87	DVNY(*)AGNNK
1262.5746	1398.7746		1398.5740 (T; 25%)	121–131	GT(*)DIEDQDLEK
			VEG		
1161.5456	1297.7456		1297.5331 (T; 50%)	21–31	AMT(°)S(°)DPEIPGK
1145.5507		1417.9507	1417.8547 (T; 25%)	21–31	AMT(*)S(*)DPEIPGK
1491.7689	1627.9689		1627.8242 (CT; 25%)	42–55	KALEGGDLEAQIT(*)F
536.3191	672.5191		672.3927 (T + CT; 50%)	84–87	RVVY(*)
557.3657	693.5657		693.4520 (T + CT; 25%)	88–92	ILPS(*)K
1199.6782		1472.0782	1472.0198 (CT; 50%)	88–97	ILPS(*)KVKDHY(*)
661.3304	797.5304		797.3954 (T + CT; 25%)	93–97	VKDHY(*)
2085.0029	2221.2029		2221.1589 (T; 50%)	138–157	GLNLDIVRPQQS(°)ET(°)CS(°)PGGN

[+] Shown are the peptides derived from analysis after enrichment by thiol chromatography (trypsin-T or chymotrypsin-CT or trypsin + chymotrypsin, T + CT) and elution at different percentages of acetonitrile: 12.5, 25, 50, and 80%). The corresponding spectra are shown in Fig. 5 (T + CT, 50%), supplementary Fig. 1 (CT, 25 and 50%), supplementary Fig. 2 (T + CT, 25%), and supplementary Fig. 3 (T, 25 and 50%)

* following Ser, Tyr, or Thr denotes mass addition at that site of 136.2 Da, indicating modification by DTT

° following Ser , Tyr, and Thr residues indicates a potential phosphorylation

peptide (21–38 and 41–50 in pOBP, 21–31 and 140–157 in VEG) suggests that several isoforms of phosphorylation co-exist for each protein. Finally, despite the combined use of two enzymes, the phosphorylation sites of peptides 59–72 in pOBP (Ser67 or Thr71) and 140–157 in VEG (Ser149 or Ser153 or Thr151) remained ambiguous. We considered the use other enzymes or chemical methods (CNBr) to assign the phosphorylation to one or the other residue, but none of these methods was suitable, as the theoretically generated peptides still contain all of the potentially phosphorylated residues. The sites could be determined by site-directed mutagenesis followed by heterologous expression in eukaryotic cells (Nagnan-Le Meillour et al. 2009), and by site mapping with the BEMAD method.

Phosphorylation of Extracellular Proteins This work demonstrated that two olfactory binding proteins (pOBP and VEG) are phosphorylated, which is a novel and unexpected result. Indeed, pOBP and VEG are secreted in the nasal mucus by the exocrine Bowman's glands via the secretion pathway. This pathway occurs in the rough endoplasmic reticulum (RER) where the signal peptide permits the translocation of proteins through the RER membrane to be processed and secreted. Thus, hypothetically these proteins are not exposed to nuclear and cytoplasmic kinases and phosphatases involved in the phosphorylation/dephosphorylation of cellular proteins. However, Jordan et al. (1994) and others have provided evidence for the existence of extracellular enzymes (ecto-enzymes) located in the extracellular space. In particular, the occurrence of ecto-kinases and ecto-phosphatases has been demonstrated in various cell types, i.e., in goat spermatozoa where they modulate sperm motility (Nath et al. 2008). Such enzymes could be responsible for the phosphorylation of OBPs after

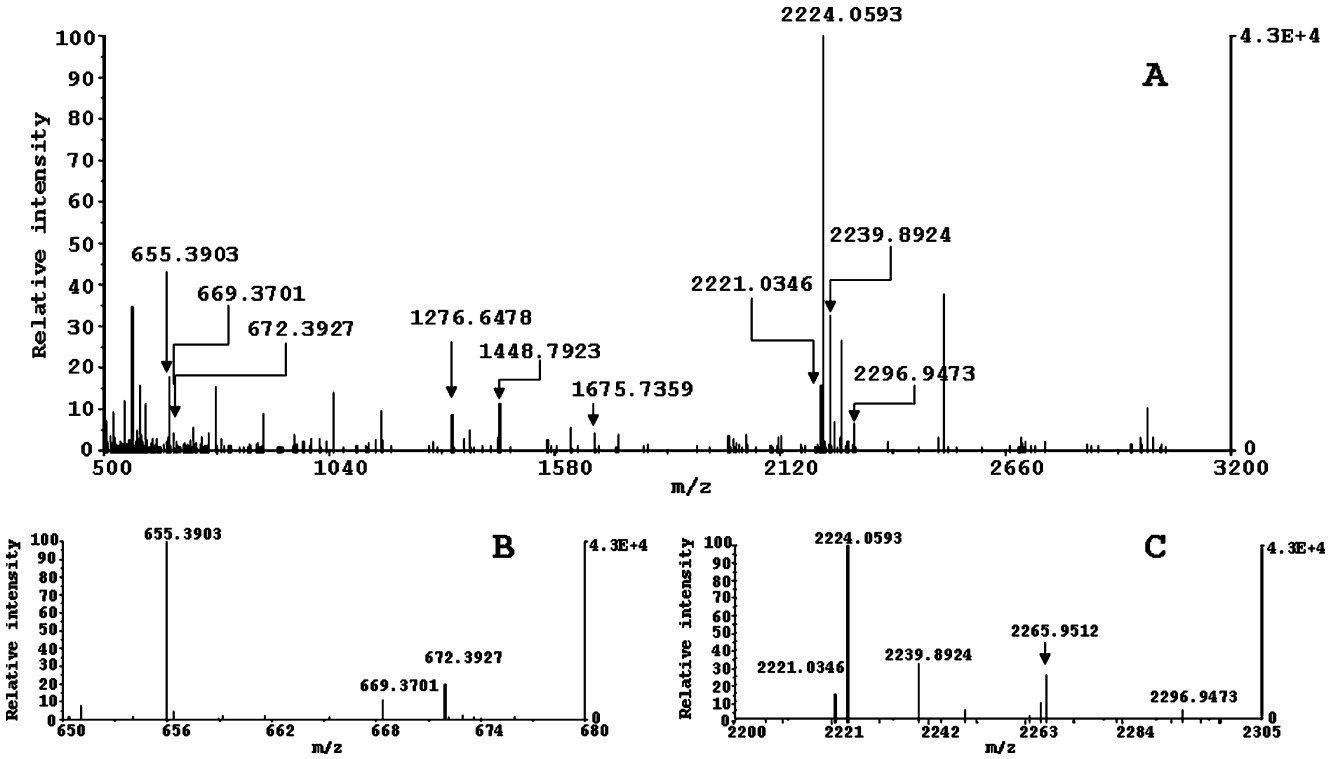


Fig. 5 Monoisotopic mass spectrum of MALDI-TOF MS analysis of carboxymethylated band containing pOBP and VEG (SDS-PAGE) of the pig, *Sus scrofa*, respiratory mucosa (RM) extract after BEMAD. **A** Peptides elution with acetonitrile 50%, after T + CT treatment. **B** Magnification of the 650–680 *m/z* zone. **C** Magnification of the 2,200–2,300 *m/z* zone. The following peaks correspond to unmodified peptides generated by the digestion of proteins: OBP (T)- 138–157 (*m/z*= 2239.8924), 138–157 Cys_CAM:155 (*m/z*=2296.9473), and 134–152 (*m/z*=2224.0593). OBP (C + CT) 51–55 (*m/z*=655.3903). VEG (T)-93–111 (*m/z*=2265.9512). DTT-modified peptides are listed in Table 2

their extracellular secretion. Hypothetically, ecto-enzymes may have the potential for intercellular regulation or reception and transduction of external stimuli. Phosphorylation is a dynamic, reversible mechanism that results in a conformational change in the structure of many enzymes and receptors, causing them to become activated or deactivated. Accordingly, the phosphorylation of pOBP and VEG creates a variety of isoforms that could possess different and specific affinities for pheromones or other odors. We recently have demonstrated that the binding specificity of the VEG1 isoform of the pig vomeronasal organ for steroids is governed by the linkage of an *O*-GlcNac moiety on the protein (Le Danvic et al. 2009). The same result was obtained with the RM tissue (data not shown). *O*-N-acetylglucosaminylation and *O*-phosphorylation can occur on the same protein and, in several instances, they map to the same (YinOYang) or adjacent sites (Comer and Hart 2000). In any case, the two modifications are dynamic and reversible, and increase the number of possible combinations for isoforms. Interestingly, Ser149 in VEG, which is phosphorylated, is also predicted by the YinOYang server (at www.expasy.org) to be *O*-GlcNac-modified. We plan to map the *O*-GlcNac sites on VEG molecules, but the results will be much more difficult to obtain than for phosphorylation, as the sub-population of glycosylated VEG is probably a minor part of the entire population. Enrichment by using affinity columns with anti-*O*-GlcNac antibody will be necessary prior to BEMAD treatment.

The existence of such variants extends the possibility of odorant coding by OBPs, strongly suggesting that this interaction is the first discrimination step, prior to the binding with olfactory receptors. The possibility of regulation of pOBP and VEG binding properties by phosphorylation and glycosylation opens new ways to study their role in perireceptor events in olfaction, as well as their genesis via biosynthesis as secreted proteins in exocrine glands.

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Mate Finding in the Parasitic Wasp *Cephalonomia tarsalis* (Ashmead): More than one way to a Female's Heart

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Abstract The parasitic wasp, *Cephalonomia tarsalis* (Hymenoptera: Bethylidae), parasitizes larvae of the saw-toothed grain beetle, *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae), and is used for biological control of this worldwide pest of stored grain. To study the hypothesis that *C. tarsalis* not only mates at its natal patch but also uses olfactory cues to find mating partners elsewhere, we investigated semiochemical use by male *C. tarsalis*. Olfactometer experiments revealed that male *C. tarsalis* are arrested by odors emanating from the cocoons of conspecifics, from young unmated females, and from feces of the host. Dodecanal, which was identified from extracts of filter paper contaminated by young females, had an arresting effect on males but not on females and was, therefore, considered as a sex pheromone. These findings indicate that *C. tarsalis* is a species with partial local mate competition. Males mate with females: 1) at the emergence site following location of females by sex pheromones from their cocoons, and 2) after dispersal from the natal patch following location of females directly by dodecanal and indirectly by unidentified sexual kairomones from host feces.

Keywords Dodecanal · Gas chromatography-mass spectrometry · Lauraldehyde · Mating strategy · Sex pheromone · Sexual kairomone

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Introduction

The parasitic wasp, *Cephalonomia tarsalis* (Hymenoptera: Bethylidae), is a specialist parasitoid of larvae of the sawtoothed grain beetle, *Oryzaephilus surinamensis* (Coleoptera: Silvanidae) (Powell 1938), and the merchant grain beetle, *O. mercator* (Fauvel). Wasps use chemical cues to locate their host larvae, attack and paralyze the hosts, and oviposit on the outside of the host. The larva feeds ectoparasitically on the host and pupates in a cocoon next to it. Because both host species are worldwide pests of stored grain and other commodities, *C. tarsalis* is used for their biological control in Europe (Zimmermann et al. 2008). However, many aspects of the wasp's biology are still unknown, and more knowledge could contribute to improve its use as a biological control agent.

One aspect that is unclear is the mating strategy of *C. tarsalis*. Females usually search for and paralyze several hosts, which they hide in a sheltered place before they begin to oviposit (Howard et al. 1998). As in many other bethylids (Griffith and Godfray 1988), this oviposition patch is aggressively defended against conspecific females (personal observations). Within the patch, females lay between one and four eggs on one host larva, depending on the number of available hosts (Powell 1938). If one egg is laid, it typically results in female offspring (Cheng et al. 2003). If more than one egg is deposited, progeny are usually of both sexes (Powell 1938). Therefore, *C. tarsalis* can be considered as a quasi-gregarious species, and most *C. tarsalis* males hatch in the vicinity of their sisters. It has been shown that these males emerge one to 2 days earlier than the females and enter their sister's cocoons for mating (Powell 1938). According to the theory of local mate competition, a strongly female-biased sex ratio in offspring would be expected in such a single foundress situation, if

all matings occur at the natal patch (Hamilton 1967). However, in *C. tarsalis*, females were found to lay one female and one male egg together on one host, i.e., to have a sex ratio of 1:1 (Powell 1938). Similarly, Cheng et al. (2003) reported a proportion of 57% females and Zimmermann et al. (2008) reported a sex ratio of 60% in large commercial breedings. Thus, the sex ratio of *C. tarsalis* seems to be low as compared to other species with local mate competition, where strong competition between males is avoided by strongly female-biased sex ratios (Thornhill and Alcock 1983), e.g., 5:1 in *Lariophagus distinguendus* (Charnov et al. 1981), 10:1 in *Nasonia vitripennis* (Werren 1980), and 4:1 in *Cephalonomia hyalinipennis* (Pérez-Lachaud and Hardy 1999). Another possible means for reducing competition between males is for not all matings to occur locally (Taylor and Bulmer 1980). This could be true in *C. tarsalis*, where 1) unmated females produce isolated sons (Powell 1938) or 2) males disperse from the natal patch after all females have been mated and have a chance to find mates in other patches (Nunney and Luck 1988). In contrast to many other bethylid species, this latter scenario is conceivable for *C. tarsalis*, since males are winged, often large and capable of dispersal. Furthermore, host- and natal patches of females often are close together due to aggregation of the host-species *O. surinamensis*, and male *C. tarsalis* live about 6 d, which seems long enough to disperse from the natal patch and find other patches. Therefore, we hypothesized that *C. tarsalis*, in contrast to many other bethylid species (Griffith and Godfray 1988) might display partial local mate competition. Under this hypothesis, some matings take place at the emergence site with siblings, and some matings occur at other patches, where non-related females emerge or are searching for hosts (Werren and Simbolotti 1989). In this case, we would expect that males use semiochemicals that are suitable to locate unmated sisters in the emergence patch, as well as semiochemicals that are suitable for the location of emerged females on other host patches (see Fauvergue et al. 1999). In order to test this hypothesis, we used bioassays to study the semiochemicals used by male *C. tarsalis* to locate its mating partners, and we used bioassays as well as coupled gas chromatography-mass spectrometry (GC-MS) to identify a female-produced sex pheromone.

Methods and Materials

Insects Specimens for cultures of *C. tarsalis* and *O. surinamensis* were collected in august 2005 from a flour-mill near Pforzheim, Germany. Cultures were kept at 23°C (*C. tarsalis*) and 27°C (*O. surinamensis*) in glass jars (height 10 cm, diam 8 cm). To rear *O. surinamensis*, approximately 500 adults were kept on a mixture of 70 g rolled oats and 100 g crimped wheat with one teaspoon of dried yeast for oviposition. The grain mixture was moistened up to 14% water content. After 7 d, the grain-mixture containing eggs and larvae was separated from adults by sieving with two sieves (2 mm and 0.63 mm mesh-size). To rear *C. tarsalis*, about 30 newly emerged wasps were placed into glass jars together with 3rd-instar larvae (3 wk-old) of *O. surinamensis*. The wasps were kept in these jars for 2 wk and were then removed. After 21 to 24 d, the next generation of parasitoids emerged and was collected every other day. To obtain samples for chemical analyses and bioassays, these parasitoids were allowed to mate within a glass vial. To obtain old mated females, these females then were allowed to oviposit on host larvae in glass jars for 2 wk. Afterwards, females were recollected and used for experiments. Cocoons and young virgin females were obtained by placing ten clean host larvae into glass-vials containing several pieces of filter paper. Mated female wasps were allowed to oviposit onto these larvae. Cocoons of developing next generation wasps were used in bioassays two weeks later. Young virgin females were obtained from cocoons 16–20 d after oviposition by daily collection from vials where no males had hatched.

To obtain information on sex ratio in our cultures, we allowed single females to oviposit on groups of 50, 100, 200, and 400 *O. surinamensis* larvae of mixed age in glass vials containing rearing medium. Offspring of *C. tarsalis* were sexed and counted afterwards. The experiment was repeated three times.

Bioassays The reaction of wasps to volatile stimuli was tested in a four-chamber-olfactometer (Steidle and Schöller 1997). This was made of heat resistant, opaque Plexiglas ® and consisted of a cylinder (height 4 cm high, diam 19 cm) divided by vertical plates into four chambers (Fig. 1). On the top of the cylinder, a walking arena (height 1 cm high, diam 19 cm) consisting of plastic gauze (mesh 0.2 mm) with a rim of heat resistant Plexiglas ® (0.9 cm high) was covered with a glass plate. No airflow was generated. A Petri dish containing an odor sample was placed beneath the arena in one chamber. The opposite chamber either contained an empty Petri dish or a Petri dish with another odor sample (see Fig. 1. T2/C3). The other chambers remained empty as transition zones. The olfactometer was illuminated from above. At the beginning of each experiment, a wasp was placed in the center of the walking arena. The behavior (walking, resting) and the position of the wasp were recorded for 420 s by using computer software "The Observer 5.0" (Noldus, Wageningen, The Netherlands). To avoid biased results due to side preferences of the parasitoids, the position of the olfactometer was rotated clockwise after every wasp. No more than ten wasps of the

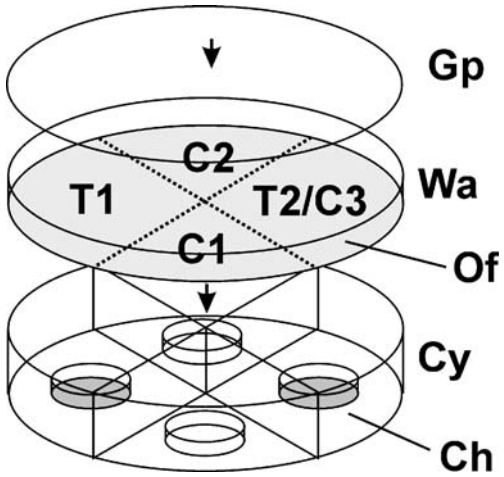

Fig. 1 Olfactometer for testing the response of wasps towards different odor sources. Height 5 cm, diam 19 cm. Abbreviations: Gp—glass plate; Wa—walking arena; Of—odor fields; Cy—cylinder; Ch—chambers; T1: test field above odor sample; C1 and C2: transition zones above empty Petri dishes; T2/C3: field opposite of T1 with odor sample in Petri-dish or with empty Petri dish

same batch were tested in one experiment, and several treatments (see below) were tested on the same day, with wasps randomly assigned to each treatment. The times that each wasp spent walking in the test and control fields of the walking arena were compared statistically by the Wilcoxon-matched pairs test by using the software package Statistica for Windows 6.0 (StatSoft Inc. 2003). All bioassays were performed with 1 to 2-d-old males (experiments 1, 3–10) or females (experiments 2, 11), which were freshly collected from rearing jars. The following samples and combinations of samples were tested:

Bioassays for Attractive Stimuli

1. To investigate the short-range response of males to cocoons, ten pupal cocoons of *C. tarsalis* (COC) were tested against the empty control (CON; *N*=25). Cocoons contained wasps 2 to 4 d before emergence.
2. To check for contamination of cocoons with larval feces, ten pupal cocoons of *C. tarsalis* (see above) were tested against the empty control. This experiment was performed with female wasps (*N*=20).
3. To test if males are arrested by feces of females hosts, 1 g rearing medium infested with *O. surinamensis*, including grain and host frass (IRM), was tested against 1 g uninfested rearing medium (URM; *N*=25).
4. To test if males are arrested by females hosts, ten 3rd-instar larvae of *O. surinamensis* (LAR) were tested against the empty control (*N*=25). To exclude contamination of host larvae with feces, the larvae were kept on filter paper for 6 hr before the experiments and were then transferred to another clean Petri dish.
5. To test if males are arrested by odors from the habitat of females hosts, ten mechanically damaged grains (MDG) were tested against empty controls (*N*=22). Mechanically damaged grains were obtained by crushing seeds with a coffee grinder.
6. To test for differences in arresting effect, five young virgin females (YVF) were tested against five old mated females (OMF; *N*=25).
7. To test if females leave male arresting residues while walking, filter paper (2.5×2.5 cm) on which ten young virgin females were allowed to walk for 15 min (FTY) was tested against filter paper on which ten old mated females had walked for the same time (FTO; *N*=25).

Bioassays for Identification of Attractive Substances Taking into account the low volatility of some of the substances and the distance of wasps from the odor source in the olfactometer, a twenty-fold higher concentration of substances was used in the bioassays than quantified from female residues (Table 1).

8. To test if fatty acids are responsible for the arresting effect of female residues on males, filter paper with a mixture of fatty acids (FAM; nonanoic acid 1.7 µg, tetradecanoic acid 6.4 µg, (*Z*)-9-hexadecenoic acid 2.9 µg, hexadecanoic acid 13.9 µg; values refer to the total amount of substance applied on filter paper in 10 µl dichloromethane) was tested against filter paper with 10 µl of dichloromethane (DCM; *N*=25).
9. To investigate if 1-tetradecene is the arresting compound, filter paper with 1-tetradecene (TDC; 24 ng total amount of substance dissolved in 10 µl of dichloromethane applied on filter paper) was tested against filter paper with 10 µl of dichloromethane (*N*=25).
10. To test if dodecanal is the arresting compound, filter paper with dodecanal (DOD; 28 ng total amount of substance dissolved in 10 µl dichloromethane applied on filter paper) was tested against filter paper with 10 µl of dichloromethane (*N*=25).
11. To test if dodecanal acts specifically on male, but not on female wasps, filter paper with dodecanal (28 ng total amount of substance dissolved in 10 µl dichloromethane applied on filter paper) was tested against filter paper with 10 µl of dichloromethane on female wasps (*N*=20).

Bioassays to Establish the Reaction Threshold To assess the reaction threshold of male *C. tarsalis* to dodecanal, either 9, 6, or 3 ng dissolved in 10 µl dichloromethane (test) and 10 µl dichloromethane (control) were applied on pieces of filter paper (2.5×2.5 cm). After solvent evaporation, the

Table 1 Compounds from residues of young virgin females on filter paper, which were absent or present in minor amounts in residues of old mated females

Number in chromatogram	Compound	Total amount released (ng/hr)
1	Nonanoic acid	87.1
2	1-Tetradecene	1.2
3	Dodecanal	1.4
4	Tetradecanoic acid	320
5	(*Z*)-9-Hexadecenoic acid	147.5
6	Hexadecanoic acid	695

filter papers were placed into a glass Petri dish and a male wasp was introduced into the Petri dish. The time of contact and the behavior of the wasp on test and control filter paper were recorded for 300 s and analyzed as described above (N=15).

Extracts To obtain extracts from female residues, pieces of filter paper (2.5×2.5 cm) were pre-cleaned by rinsing with dichloromethane. After solvent evaporation, either ten young virgin females (see above) or ten old mated females (see above) were allowed to walk on the filter paper for 1 h. Subsequently, filter papers were rinsed with 2 ml of dichloromethane. Extracts were concentrated to 500 µl in a water bath at 60°C. Before chemical analysis, each extract was checked for an arresting effect on males (N=7–10). Attractive extracts were further concentrated to 50 µl for chemical analyses (N=3).

To obtain extracts from cocoons, ten cocoons containing wasps 2 to 4 d prior to emergence were extracted in 1 ml of dichloromethane for 1 h. The extract was filtered and concentrated to 50 µl in a water bath at 60°C for chemical analyses (N=3).

Chemical Analyses Extracts were analyzed by coupled gas chromatography-mass spectrometry (GC-MS) employing a 6890 GC gas chromatograph linked to a 5973 N MSD (both Agilent Technologies, Santa Clara, CA, USA) in electron impact mode at 70 eV with helium as carrier gas (1.2 ml/min). Separations were done by using an HP-5MS column (30 m, 0.25 mm i.d., 0.25-µm film thickness; Agilent) that was operated at 60°C for 3 min, increased to 300°C at a rate of 3°C /min, and finally held at this temperature for 10 min. Both the injector and the transfer line between GC and MS were heated to 250°C. For identification, mass spectra and retention times of candidate peaks were compared to mass spectra and retention times of commercially available standard compounds.

Comparison of Substances from Old Mated and Young Virgin Females Extracts of trails from old mated females and young virgin females were compared to an extract of clean filter paper. Peaks occurring in the extract of clean filter paper were excluded from the analysis. Remaining peaks were compared semi quantitatively by using relative peak areas. Each peak analyzed in a sample was standardized by dividing its area by the total of analyzed areas in the sample with Primer 6 software package (Primer-E Ltd. 2006). Mean percentages of peak areas from old mated females were subtracted from mean percentages of peak areas from young virgin females. Peaks with mean peak area higher in extracts from young females were identified, and substances were tested in bioassays for arrestment. The threshold was set at 0.5% difference in relative peak area.

Quantification of Potential Semiochemicals To quantify substances from female residues on filter paper, 100 ng of synthetic substances were applied on pre-cleaned filter paper. After 1 h, substances were recovered by rinsing the filter paper with 1 ml dichloromethane and analyzed by GC-MS. Resulting peak areas were compared to peak areas obtained from female trails after females were allowed to walk on filter paper for 1 h (N=3). To assess the amount of dodecanal found on filter paper after the presence of single females within a shorter period of time, females were allowed to walk on filter paper for 15 min. The filter paper was analyzed and dodecanal was quantified as described above.

Chemicals Chemicals and solvents were purchased from Sigma-Aldrich (Munich, Germany) or Merck (Darmstadt, Germany) and were of highest purity available.

Results

Breeding experiments of *C. tarsalis* revealed an actual sex ratio of 1: 2.29 ± 1.09 (Mean ± s.d.; male: female) in our cultures. There was no correlation between sex ratio and host availability (Spearman rank r=0.00, N=15, P=1.0).

Silk cocoons of *C. tarsalis* containing wasps 2 to 4 d before emergence, which had developed in the absence of grains and host feces, had an arresting effect on male wasps (Fig. 2a). Female wasps, which were tested to control for the contamination of the cocoons with host feces, did not react to the cocoons (Fig. 2b). In addition, male wasps were

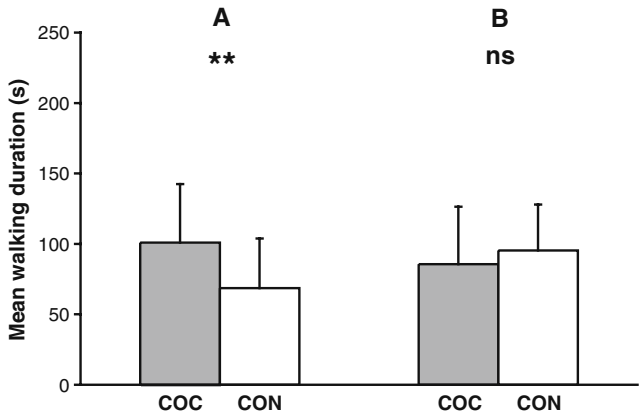

Fig. 2 Walking time (mean ± s.d.) of male (**a**) and female (**b**) *Cephalonomia tarsalis* in the odor field of an olfactometer with ten pupal cocoons of *C. tarsalis* and an empty control field. Abbreviations: COC—cocoons; CON—empty control; ns—not significant; ** $P<0.01$ (*Wilcoxon-matched pairs test*)

strongly arrested by the infested rearing medium of *O. surinamensis* and significantly preferred the respective olfactometer field to the field with uninfested rearing medium (Fig. 3a). Mechanically damaged grains (Fig. 3c) as well as larvae of *O. surinamensis* (Fig. 3b) showed no arresting effect on males of *C. tarsalis*. Therefore, host feces are the component in the grain-host-complex that induces arrestment.

Young virgin females had an arresting effect on males when old mated females were placed in the opposite olfactometer field (Fig. 4a). Similarly, filter paper that had been walked on by young virgin females was highly arresting when compared to filter paper that had been walked on by old mated females (Fig. 4b). Extracts of these filter papers with dichloromethane analyzed by GC-MS

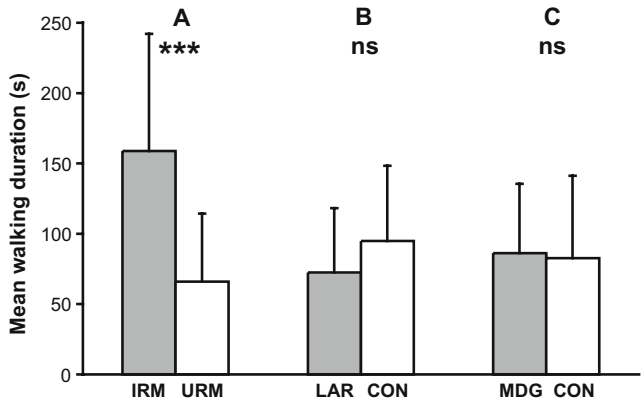

Fig. 3 Walking time (mean ± s.d.) of male *Cephalonomia tarsalis* in the odor fields of an olfactometer with the potential kairomone sources **a** infested rearing medium, **b** uninfested rearing medium and **c** ten 3rd instar larvae of *Oryzaephilus surinamensis* and an empty control field. Abbreviations: IRM—infested rearing medium; URM—uninfested rearing medium; LAR—larvae; CON—empty control; MDG—ten mechanically damaged grains; ns—not significant; *** $P<0.001$ (*Wilcoxon-matched pairs test*)

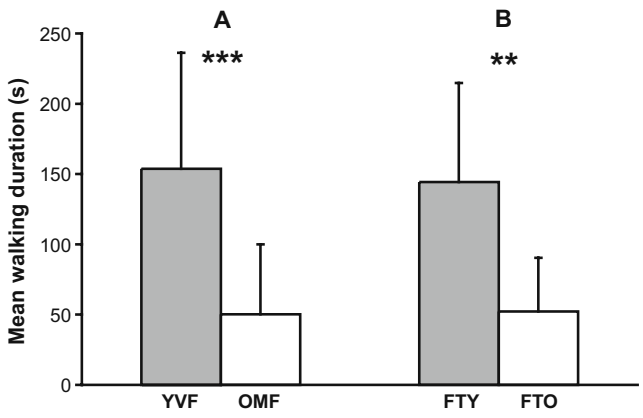

Fig. 4 Walking time (mean ± s.d.) of male *Cephalonomia tarsalis* in the odor fields of an olfactometer with the potential pheromone sources **a** five young virgin females and five old mated females of *C. tarsalis* and **b** residues on filter paper from ten young virgin females and residues on filter paper from ten old mated females of *C. tarsalis*. Abbreviations: YVF—five young virgin females; OMF—old mated females; FTY—residues from young virgin females; FTO—residues from old mated females; ** $P<0.01$; *** $P<0.001$ (*Wilcoxon-matched pairs test*)

revealed six compounds that occurred in higher amounts in extracts from young as compared to old females (Fig. 5, Table 1). Based on the comparison of mass spectra and retention times, these compounds were identified as four fatty acids (nonanoic acid, tetradecanoic acid, (*Z*)-9-hexadecenoic acid, and hexadecanoic acid), 1-tetradecene, and dodecanal. Neither a mixture of the four fatty acids

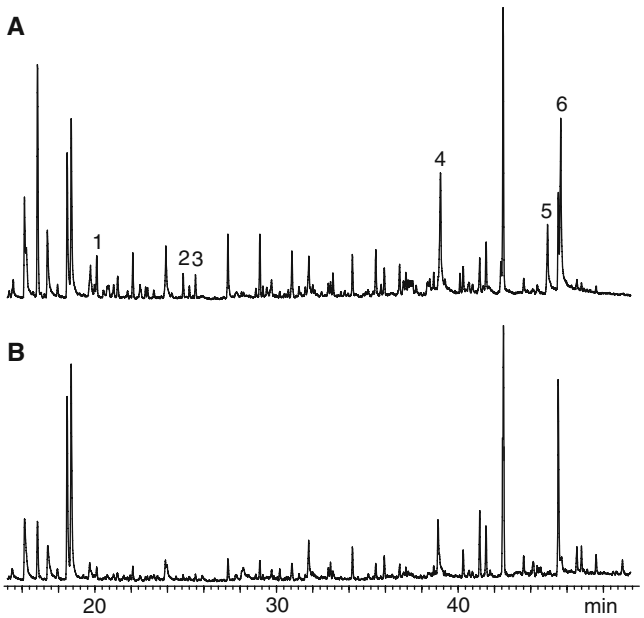

Fig. 5 Representative total ion chromatograms of extracts of residues of ten young virgin females (**a**) and ten old mated females (**b**) of *Cephalonomia tarsalis*. The numbers in the chromatograms refer to the numbers of the compounds in Table 1. (30 m HP—5MS capillary column, 3 min at 60°C, then 3°C/min to 300°C)

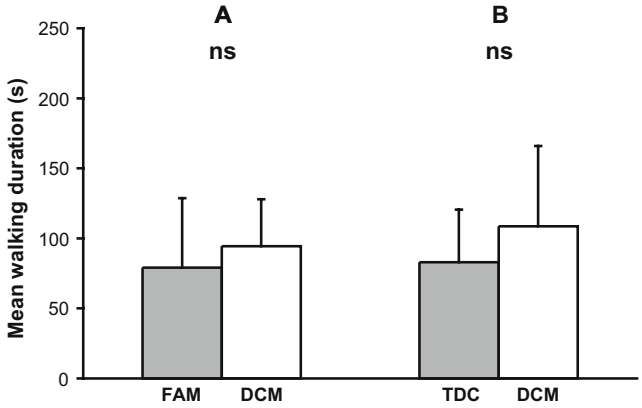

Fig. 6 Walking time (mean ± s.d.) of male *Cephalonomia tarsalis* in the odor fields of an olfactometer with synthetic compounds of *C. tarsalis* residues on filter paper. (**a**) 10 µl of a mixture of four fatty acids in dichloromethane and (**b**) 10 µl of 1-tetradecene in dichloromethane and control fields with dichloromethane. Abbreviations: FAM—fatty acid mixture; DCM—10 µl dichloromethane; TDC—1-tetradecene; ns—not significant (*Wilcoxon-matched pairs test*)

(Fig. 6a) nor 1-tetradecene (Fig. 6b) arrested male wasps in the olfactometer. In contrast, dodecanal significantly arrested males (Fig. 7a). Females did not react to dodecanal (Fig. 7b).

Experiments to quantify the amount of dodecanal on filter paper after female visits revealed that 15 min of contact by one female results in the deposition of about 3 ng dodecanal on the filter paper. Experiments where wasps had direct contact to dodecanal on filter paper demonstrated that 6 ng of dodecanal were sufficient to obtain a significant arrestment response in male *C. tarsalis*, whereas 3 ng did not elicit a significant reaction (Fig. 8). Dodecanal was present only in trace amounts in cocoons 2 to 4 d prior to wasp emergence.

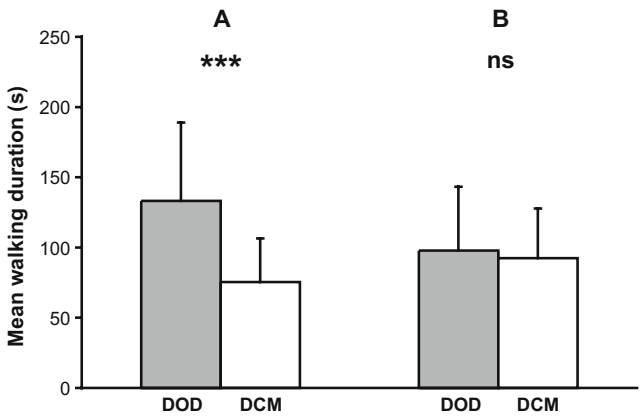

Fig. 7 Walking time (mean ± s.d.) of (**a**) male and (**b**) female *Cephalonomia tarsalis* in the odor field of an olfactometer with 10 µl of dodecanal in dichloromethane and the control field containing dichloromethane. Abbreviations: DOD—dodecanal; DCM—dichloromethane; ns—not significant; *** $P<0.001$ (*Wilcoxon-matched pairs test*)

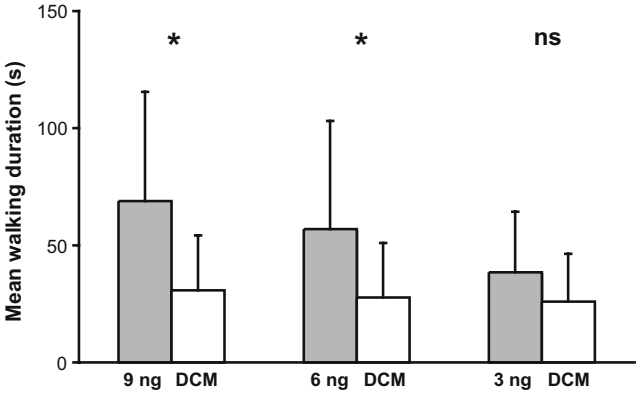

Fig. 8 Walking time (mean ± s.d.) of male *Cephalonomia tarsalis* on filter paper treated with 9, 6, and 3 ng of dodecanal in 10 µl dichloromethane and the filter paper with dichloromethane. Abbreviations: 9 ng–9 ng dodecanal; 6 ng–6 ng dodecanal; 3 ng–3 ng dodecanal; DCM—dichloromethane; ns—not significant; *$P<0.05$ (*Wilcoxon-matched pairs test*)

Discussion

This study revealed that males of the parasitic wasp *C. tarsalis* are significantly arrested in the olfactometer by odors that originate from cocoons containing conspecifics up to a distance of several centimeters. This result supplements earlier studies that showed that males of *C. tarsalis* were arrested when in direct contact with cocoons (Howard 1998). It is likely that these odors emanating from cocoons are used for mate location. To test if feces contamination from the host larvae in the cocoons might be responsible for the arrestment, we also studied the response of female wasps to the cocoons. Earlier studies revealed that host feces have a strong arresting effect on females (Collatz and Steidle 2008). In these control experiments, females did not react to the cocoons. Thus, it is unlikely that the cocoons are contaminated with feces from the host larvae. To check if dodecanal (see below) might be responsible for the reaction of males to conspecific cocoons, cocoons were chemically analyzed. Dodecanal was found only in minimal trace amounts and, therefore, is likely not responsible for the attractiveness of cocoons. Thus, the identity of the behavioral chemical or chemicals that emanate from the cocoons bears further study.

In addition to female cocoons, males of *C. tarsalis* were arrested in the olfactometer by young virgin females as well as by filter paper that had been walked on by young virgin females. These young females likely release arresting volatiles, which are either absent or released in lesser amounts by old females. Because young virgin females were compared to old mated females, it remains unclear if mating status or age are responsible for the difference. However, males do not prefer virgin to mated females of the same age, and females mate several times within 24 h

after emergence (Cheng et al. 2004). Therefore, it is reasonable to assume that the difference is due to age rather then to mating status of the wasps.

Chemical analyses revealed several compounds that were present in larger amounts in the residues of young females on filter paper as compared to old females. Of these, only dodecanal had an arresting effect on male, but not on female wasps in the olfactometer. The amount of dodecanal released by a single female per 15 min was around 3 ng, and behavioral experiments revealed a reaction threshold by male *C. tarsalis* to dodecanal between 3 and 6 ng. Thus, considering analytical constraints due to the low amount of substance, the male reaction threshold is within the same range as the amount of dodecanal released by one female. Therefore, we suggest that dodecanal is a sex pheromone *sensu* Landolt and Phillips (1997), which acts specifically on the behavior of the opposite sex (males) and enables mate finding. It is unclear so far, if dodecanal is deposited on the ground as a trail pheromone or released in the air and subsequently adsorbed on the filter paper. According to its chemical nature, dodecanal would be suitable as a trail pheromone. Its volatility is low enough to remain on the substrate for a certain time but also high enough to form an odor-space above the substrate. Sexual trail pheromones are known from other parasitoid species, e.g., *Aphytis melinus* (Bernal and Luck 2007), *Trichogramma brassicae* (Pompanon et al. 1997), and *Aphelinus asychis* (Fauvergue et al. 1995). However, to our knowledge, none of the parasitoid trail pheromones has yet been identified.

Interestingly, substrate-borne semiochemicals are not only used by males of *C. tarsalis* for mate location, but by also by females for host finding. Chemical cues released by wandering host larvae of *O. surinamensis* arrest female wasps only for up to 30 min, thus adding a time-specific component to the host-finding process of *C. tarsalis* (Collatz and Steidle 2008). We hypothesize that a similar mechanism is involved with the female sex pheromone of *C. tarsalis*, especially when considering the tight agreement between reaction threshold and amount of pheromone released.

In addition to sex pheromones from cocoons and female residues, males of *C. tarsalis* also reacted positively to odors from feces of the host *O. surinamensis*. Odors from the host (in absence of feces) and from mechanically damaged grains (i.e., the host habitat) had no effect on male response. Because female *C. tarsalis* also use odors of host feces to locate their hosts (Collatz and Steidle 2008), these odors guide males to the female's oviposition site and thus agree with the definition of a sexual kairomone *sensu* Ruther et al. (2002). According to this definition, a sexual kairomone is composed of one or more allelochemicals, which are used by the benefiting organism for sexual purposes. The use of host kairomones in parasitoids for mate finding is known from several parasitoid species. For example, males of the parasitic wasp *Lariophagus distinguendus* use volatiles emitted by the larvae of its host, the granary weevil, *Sitophilus granarius* (Steiner et al. 2007). In the parasitoid *Venturia canescens*, female pheromones were three times more attractive to males when they were combined with host-related odors, whereas the latter were not attractive by themselves (Metzger 2008).

Taken together, males of *C. tarsalis* use semiochemical cues from different sources to locate females at different sites for mating. They use sex pheromones released by conspecific cocoons to locate sisters at the emergence site; they react to dodecanal, a female sex pheromone to locate females over some distance; and they use sexual kairomones from the feces of the host to find host-feeding and oviposition sites of females away from the emergence site. These findings support the hypothesis that in contrast to other bethylids, *C. tarsalis* is a species with partial local mate competition, with matings at the emergence site and matings after dispersal from the natal patch. This might explain the observed sex ratio of one male to 2.29 females in *C. tarsalis*, which is lower than the more female-biased sex ratios of other parasitic wasps, where local mate competition is more pronounced.

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A Two-Component Female-Produced Pheromone of the Spider *Pholcus beijingensis*

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Abstract Chemical signaling plays an important role in spider sexual communication, yet the chemistry of spider sex pheromones remains poorly understood. Unlike insects and mammals, the identification of spider pheromones has seldom been attempted, and no multicomponent pheromones have been found. Empty webs of sexually receptive females of *Pholcus beijingensis* were more attractive to male conspecifics as compared to webs of sexually unreceptive females or to mature males. Coincidently, chemical analysis revealed that (*E,E*)-farnesyl acetate, diisobutyl phthalate, and hexadecyl acetate of the spider webs exhibited higher relative abundances in sexually receptive females than in sexually unreceptive females or males, indicative of possible pheromone components. Two-choice behavioral assays verified that the blend of (*E,E*)-farnesyl acetate and hexadecyl acetate (w/w: 2:1) attracted males at a dosage equivalent to the amounts of these compounds in one spider web, whereas neither compound alone aroused males. In addition, diisobutyl phthalate (a likely contaminant from contact with plastic) alone or in combination with either of the acetates did not evoke the males' attraction. The behavioral data suggest that (*E,E*)-farnesyl acetate and hexadecyl acetate comprise a two-component female-produced sex pheromone in *P. beijingensis*, the first multicomponent pheromone found in spiders.

Keywords (*E,E*)-Farnesyl acetate · Hexadecyl acetate · Sex pheromone · Spider web · Araneae · Pholcidae

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Introduction

Spiders generally are aggressive and cannibalistic. They have evolved elaborate means to communicate with each other during courtship. The exchange of chemical signals is probably the first type of communication in spiders that bring the males and females together (Weygoldt 1977). Chemical signaling plays an important role in spider sexual communication, and contact between the emitter and the receiver is not necessary (Gaskett 2007). Silk-bound pheromones are important in courtship. For example, in some species, a mate-searching male rapidly cuts the thread of a virgin female's web or packs the silk into a tight mass. This behavior may hinder evaporation of the sex pheromone thereby reducing the chances of another male reaching the web (Watson 1986; Schulz and Toft 1993). Male wolf spiders, *Schizocosa ocreata,* show significantly more and longer bouts of chemosensory and courtship behaviors on the silk of adult, unmated female spiders than on any other stimulus treatment (Roberts and Uetz 2005).

Silk-bound sex pheromones occur in several arachnid families (Dondale and Hegdekar 1973; Suter and Renkes 1982; Searcy et al. 1999; Michael and Maydianne 2004; Roberts and Uetz 2004, 2005). However, the chemistry of these remains poorly understood, especially in comparison with those of crustaceans and insects, and some mammals,

reptiles, and fishes. There are only three species of spiders for which pheromones have been identified. The pheromone compounds (*R*)-3-hydroxybutyric acid (HBA) and its dimer, (*R*)-3-[(*R*)-3- hydroxybutyryloxy]-butyric acid (HBBA), occur in a sheet-web spider *Linyphia triangularis* (Schulz and Toft 1993). The wandering spider, *Cupiennius salei*, and the orb-web spider, *Agelenopsis aperta*, have two other sex pheromones: (*S*)-1,1′-dimethyl citrate (Papke et al. 2000; Tichy et al. 2001) and 8-methyl-2-nonanone (Papke et al. 2001). Structurally unrelated to other known pheromones, both HBA and (*S*)-1,1′-dimethyl citrate seem to be unique to spiders. The pheromone 8-methyl-2-nonanone resembles a component of insects, that include the caddisfly, *Hesperophylax occidentalis,* and the Asian palm weevil, *Rhynchophorus ferrugineus* (Hallett et al. 1993; Bjostad et al. 1996). Herein, we explored the structure, function, and chemical mechanism(s) of sexual communication in spiders.

Pholcid spiders (Araneae, Pholcidae) often are found in houses and buildings, especially in basements and cellars. *Pholcus beijingensis* is common in various caves in the vicinity of Beijing. They usually spin untidy webs in corners or on the stone walls of cave entrances. Sexual selection occurs in pholcids (Uhl 1998; Uhl et al. 2005). The species is polygamous, and males and females alike have multiple mating partners. During the reproductive season, males abandon their webs to search for potential mates, while the females wait on their webs for males. The approach of a mature male rarely triggers predatory or aggressive behavior in sexually receptive females. In natural populations, immature and adult spiders overlap during much of the active breeding season (Chen and Li 2005; Chen et al. 2008). Selection may favor males that distinguish conspecific, sexually receptive females during the reproductive season, otherwise they would be eaten. We isolated chemical signals from the web thread of *P. beijingensis*, and identified the sex pheromone components.

Methods and Materials

Subjects

Juvenile and adult *P. beijingensis* were collected in March and May of 2007 at the entrance of a bat cave located southwest of Beijing, China (39º42.350′N, 115º42.825′E). Each spider was kept in a glass cuvette (4 cm i.d.×12 cm high) with a small moistened wad of cotton on the bottom to provide humidity. Cuvettes were put in a climatic chamber (RXZ-268B, Ningbo Jiangnan Instrument Factory) under a 14:10 h (L/D) photoperiod regime at 25°C (day) and 22°C (night). About 10–15 fruit flies (*Drosophila melanogaster*) were provided to each spider for food once a week.

Test for Attraction to Silk of Females

We screened the following classes of *P. beijingensis*: juveniles, sexually receptive females, egg-holding females, and mate-searching males. To determine whether the adults were reproductively active or not, we paired each male with an adult female on the female web, and checked for courtship behavior (Table 1). We removed the male from the web when it unfolded its pedipalps to the female. These sexually receptive females and males were chosen for the experiments. Egg-holding mothers were taken as sexually unreceptive females because they always held their egg sac with their chelicerae until the spiderlings hatched; generally, they remained sedentary and would not copulate with males during this phase. We randomly paired 30 females with 30 males until mating occurred. Thus, we also obtained gravid spiders.

We tested the hypotheses that the silk of sexually receptive females was attractive to mate-searching males by using a two-choice arena composed of three quadrates consisting of plastic chambers (upper dimensions: 16.5×11 cm; lower: 14.5×9 cm, height: 5.5 cm), each of which component was removable (Fig. 1). A hole was cut into one side of each choice chamber, and in both ends of the release chamber. A selecting male was released into the central chamber and could move freely into the left or right choice chamber.

We completed three sets of trials. The silk of sexually receptive females was presented in all sets, while either silk from juvenile spiders, silk from mate-searching males, or silk from egg-holding females was presented as the alternative choice. Male *P. beijingensis* were introduced into the central release chamber and allowed to acclimate to the surroundings for 1 h before the trials. The silk stimuli consisted of empty webs (no spider present) woven by test spiders. The order of treatments in the arena system was randomized. All male attraction tests were completed at night (usually from 20:00 to 22:00 h). We observed movement of the selecting males under infrared light, and recorded which chamber it first chose during a 2 h observation period. We deleted trials when a selecting male was disturbed and escaped into a choice chamber as soon as the choice arena system was connected. Each selecting male was used in only one trial of one test. A minimum of 15 males was tested in each trial set.

We also tested the attractiveness of extracts of silk from females. Pheromone from the silk of sexually receptive females was extracted with dichloromethane (5 µl for each web). A piece of filter paper containing the extract was placed in one of the choice chambers; the other choice chamber contained a filter-paper with an equal quantity of dichloromethane only. The choice chambers were occupied alternatively with the silk extract and the solvent only. Selecting males were allowed 2 h to discriminate between the silk extract and the solvent treatments.

Table 1 Sequence of courtship behavioral patterns displayed in the spider, *Pholcus beijingensis*

Behavior pattern	Description
Level 1	**Detection and location (♂)**
Pedipalps waggle	Shaking pedipalps up and down
Abdomen vibration	Shaking abdomen, usually immediately following pedipalps shaking
Adjust position	Picking up legs and shuffling body within same location
Search	Shoot jerky movements on web with frequent direction shifts and then turn towards the female
Level 2	**Signaling and approaching (♂)**
Flex	Slow or quick push on web with walking legs while raising body, followed by leg retraction with body lowering to make vibration of the web
Body tremor	High frequency dithering on the web to cause shoot and rapid quiver of the web during walk
Approach	Walk towards female spider with a few interruption for rubbing walking legs with his chelicerae
Pluck web	Short pull on silk threads with one of front legs alternatively in front of the female till she reacts (e.g. plucking back, adjusting direction) alternately, with rarely touching the female legs
Level 3	**Preparation and mate initiation (♂ & ♀)**
Leg spread	Male faces female from a distance of < 2 cm, with femurs of all walking legs spread backward in a line, forming a right angle with the web
Pedipalp unfolding	Male unfolds pedipalps up and forwards with abdomen cocked and legs spread backward, keeping this posture and waiting for approaching female
Contact	Female approaches male, both female and male contact each other with front part of cephalothorax or first pair of walking legs and frequently adjust body position
Mate initiation	Male clamps female's epigynum with his chelicerae and his two pedipalps inserts into the female's genital pore, at the same time female raises all her walking legs together to support her abdomen.

Levels refer to temporal stages within the courtship sequence from transition analysis of *Agelenopsis aperta* presented by Singer (Singer et al. 2000) and *Pholcus beijingensis* presented by Chen (Chen and Li 2005).

Silk Sample Collection and Extraction

Sexually receptive females, mate-searching males, and sexually unreceptive female spiders (egg-holding females) supplied silk samples. Square boxes made of cardboard (22 cm l.×22 cm w.×8.5 cm h.) were used for the spiders to weave webs. A glass Petri dish with cotton soaked in distilled water was set in each box to supply humidity. All boxes were cleaned with alcohol (99%) and air-dried before use. We released the spiders into the boxes individually.

Fig. 1 Two-choice arena system used in assessment of the attraction of the silk and the potential pheromones. The three chambers were upended and spliced with a hole on one or two sides. The selecting male was released in the central chamber and could move freely to the left or right choice chamber

After 48 h, the web silk was curled into a very small ball and put into a glass capillary (1.8 mm i.d.×3 cm l.), which had been fused at one end. The capillary containing silk was then sealed in a screw-cap vial (Agilent Technologies, USA). Compounds from each silk sample were extracted with 5 μl dichloromethane (purity > 99.5%, Beijing Fine Chemical Company, Ltd., Beijing, China) for 48 h. Following extraction, we removed the silk, and stored the remaining solution at −20°C until analysis by gas chromatography-mass spectrometry (GC-MS).

GC-MS Analysis

Analytical GC-MS was performed on an Agilent Technologies Network 6890N GC system coupled with 5973 Mass Selective Detector with the NIST/EPA/NIH Mass Spectral Library (2002 version; Agilent Technologies 2002). Chemstation software (Windows 2000) was used for data acquisition and processing. The GC was equipped with a 30 m HP5-MS capillary column (0.25 mm i.d.×0.25 μm film thickness). Helium was used as the carrier gas at a flow rate of 1.0 ml/min. The temperature of the injector was set at 280°C. One μl of sample was injected in the splitless mode. The oven temperature was programmed from 80°C to 240°C at 5°C/min. Then, the temperature was increased

by 10°C/min up to 280°C and held for 10 min. Electron impact ionization used 70 eV, and the scanning mass ranged from 30 to 450 amu. Compounds were identified tentatively by matching their gas chromatographic retention times and mass spectra with authentic analogs of the mass spectral library. (*E,E*)-Farnesyl acetate (FA, 95%; Sigma-Aldrich, Inc., St. Louis, MO, USA), diisobutyl phthalate (DIBP, 99.0%; Laboratories of Dr. Ehrenstorfer GmbH, Germany) and hexadecyl acetate (HA, 95%; Sigma-Aldrich, Inc., St. Louis, MO, USA) were used to confirm identification of unknown products after separation on a non-polar column (HP5-MS) and a polar column (HP-INNOWax, 30 m long, 0.25 mm i.d. × 0.25 μm film thickness).

Statistical Analysis

All statistical analyses were conducted using SPSS for Windows (version 15.0; SPSS Inc. 1999). We formed two hypotheses to test statistically. The first stated that no sexual dimorphism occurred in the relative abundances of crude extract compounds from webs. The second stated that there was no difference in the relative abundances of the compounds in the crude extracts from receptive females and sexually unreceptive females. To test the hypotheses, we measured the relative abundance of each compound by converting the peak area of a particular compound into a percentage of the sum peak areas from the 14 main GC peaks. If a given GC peak was too small to display the diagnostic MS ions, which rarely occurred, its area was taken as zero. Subsequently, the relative abundances of the compounds were analyzed by using either parametric tests, when the data were normally distributed, or non-parametric tests, when the data were not normally distributed. To analyze differences in the relative abundances of the compounds, we used Mann–Whitney *U* test for compound 8 when comparing differences between sexually receptive females and mate-searching males, and compound 14 in comparing differences between sexually receptive females and egg-holding females, which did not have normally distributed raw data. Other compounds were analyzed with an independent two-tailed *t* test when the raw data were normally distributed. The Chi-square test was used to analyze observed counts to the expected counts for male choice data.

Titer Analysis of Putative Pheromone Components

We determined the content of each putative pheromone component on a female's web. FA, HA, and DIBP each were diluted sequentially in dichloromethane to concentrations of 0.0001, 0.001, 0.01, 0.1, and 1 μg/μl. We injected 1 μl of each prepared solution into the GC-MS. GC detection showed that the peak areas of these compounds in female silk extract were close to those of the test samples at 0.01 μg/μl. Therefore, we injected 1, 2, 3, 4, and 5 μl of authentic sample at 0.01 μg/μl in GC to obtain the calibration regression equation. The quantity of each putative pheromone component on a female's web was calculated by comparing the peak area with that of the synthetic standard sample.

Test for Biological Activity of the Potential Pheromone Components

Bioassay of the potential pheromone components involved three steps. First, attractiveness of each compound to mate-

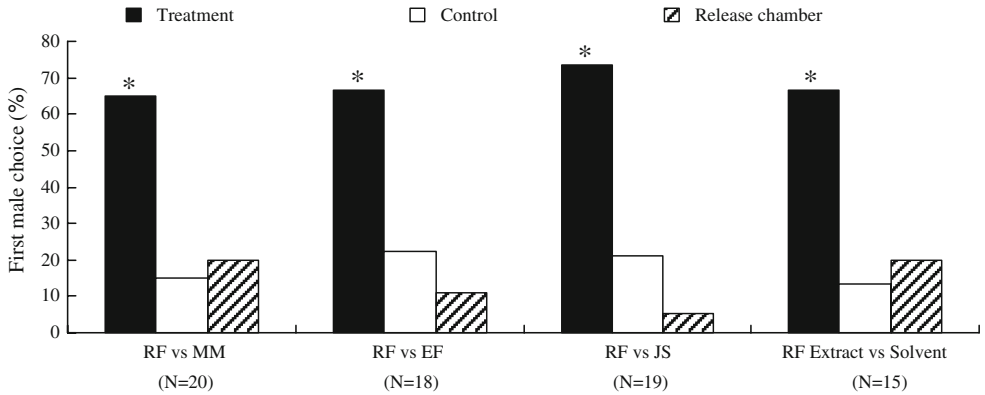

Fig. 2 Results of male *Pholcus beijingensis* attraction to sexually receptive female silk and the silk extract. Trials were completed in the two-choice arena system (Fig. 1). *RF* web of the sexually receptive female; *MM* web of the mate-searching male; *EF* web of the egg-holding female; *JS* web of the juvenile spider; *RF Extract* silk extract of the sexually receptive female; *Solvent* an equivalent amount of dichloromethane to the silk extract. The catalogue 'treatment' refers to sexually receptive female webs in all trials; 'Control' refers to webs of other spiders including mate-researching male, egg-holding female, and juvenile spider in corresponding trials; 'Release chamber' refers to males that within the 2 h observation period failed to leave the central arena into which they had been introduced 1 h before the start of the trial. * indicates $P < 0.05$

searching males was tested in the two-choice arena. Second, attractiveness of the tertiary blend involving FA, HA, and DIBP was tested. Third, we tested binary blends to check whether attractiveness to males was retained as follows: FA and DIBP; FA and HA; and HA and DIBP. The relative quantity of each component in all trials was equivalent to that naturally contained in a female's web.

Because the blend of FA and HA was significantly attractive to males, we diluted FA and HA in dichloromethane to concentrations of 0.001, 0.01, 0.1, and 1 μg/μl, and we used 10 μl FA and 5 μl HA of the series to determine the attractiveness of differing concentrations. An equivalent amount of dichloromethane was applied as the solvent control. We observed the movement of the males under infrared light for 2 h, and recorded which chamber they chose first.

We also determined whether the pheromone would elicit male courtship. We placed a filter-paper with the pheromone (10 μl FA and 5 μl HA at a concentration of 0.01 μg/μl) on an empty egg-holding female web, and then introduced a male. We observed each male for 2 h to detect whether the male displayed courtship signaling or not. We also observed male mate-searching behavior on empty webs produced by sexually receptive females.

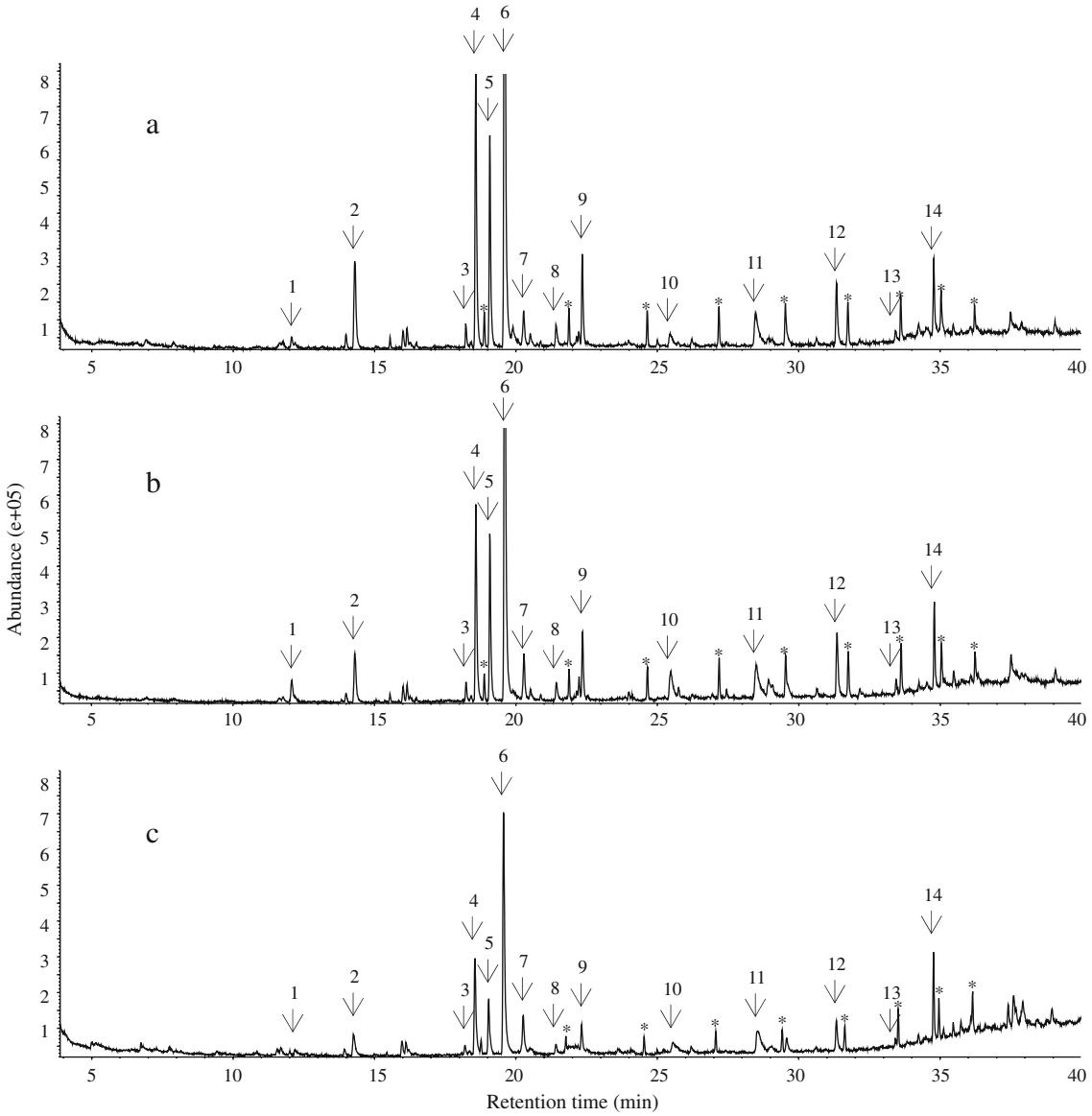

Fig. 3 Representative gas chromatograms of the crude extract of sexually receptive female silk **a**, mate-searching male silk **b** and egg-holding female **c**. The numbers that label the GC peaks correspond to peak numbers in Table 2. * denotes the Si-containing compounds that are regularly presented in the chromatograms. These silaceous compounds were not considered as potential pheromone components but contaminants from the GC column

Results

Male Attraction to Sexually Receptive Females' Silk and Silk Extract

The results of the two-choice arena trials are presented in Fig. 2. Adult male *P. beijingensis* were significantly attracted to webs produced by sexually receptive females (Chi-square test: X_1^2=6.250, P<0.05; X_1^2=4.000, P<0.05; X_1^2=5.556, P<0.05, respectively; Fig. 2). Dichloromethane extract of the silk from sexually receptive females also attracted mate-searching males (X_1^2=5.333, P<0.05; Fig. 2). The selecting males moved from the release chamber directly into the chamber previously occupied by sexually receptive females, and most males would stay on the empty females' webs until the next morning.

Identification of Potential Pheromone Components

More than 20 different compounds were regularly detected (some in low quantities) in silk samples of *P. beijingensis*, including some silaceous compounds. These were present in all samples and are considered most likely to be contaminants from the column; therefore, they were dismissed (Fig. 3).

We tentatively identified 14 compounds detected in the silk samples by matching GC retention times and mass spectra with analogs in the mass spectral library (Table 2). No qualitative differences were observed in chromatograph peaks among the silk extracts of the sexually receptive and egg-holding females and the mate-searching males. Egg-holding females usually spun less silk than sexually receptive females and mate-searching males. The total peak area detected from the silk extract of egg-holding females was less than those obtained from the other sample groups.

Quantitative analyses of relative abundances of the relevant compounds are presented in Table 2. Relative areas of peaks 5, 6, and 9 occurred in significantly greater proportions in silk extracts of the sexually receptive females than those of mate-searching males. These compounds also displayed significant differences between receptive females and egg-holding females, and they were less abundant in the silk extract of egg-holding females (Table 2). Therefore, they were considered as putative pheromone components of *P. beijingensis*. The mass spectra (Fig. 4) matched those of synthetic standards following separation with non-polar and polar columns. Thus, compounds 5, 6, and 9 were (*E,E*)-farnesyl acetate (FA), diisobutyl phthalate (DIBP), and hexadecyl acetate (HA), respectively.

Table 2 Comparison of relative abundance of compounds in silk extract of the spider *Pholcus beijingensis* (Mean ± *SD*)

Peak No.	Retention Time (min)	Compounds	Relative Abundance (%)			Statistical Significance (*P*)	
			RF (*N*=8)	MM (*N*=8)	EF (*N*=9)	RF *vs* MM	RF *vs* EF
1	12.06	Butylated hydroxytoluene	0.44±0.25	2.01±0.92	0.72±0.71	0.002	0.301
2	14.31	Tetradecanal	3.30±1.99	4.58±1.13	5.98±1.51	0.136	0.007
3	18.23	Octadecane	0.98±0.33	0.91±0.27	1.25±0.39	0.633	0.140
4	18.58	Hexadecanal	9.11±4.77	12.25±2.88	16.20±4.72	0.133	0.008
5[c]	19.08	*E,E*-farnesyl acetate [a]	11.28±2.91	8.02±1.11	1.74±1.36	0.010	0.000
6[c]	19.60	Diisobutyl phthalate [a]	44.65±11.66	32.73±3.74	10.56±6.12	0.024	0.000
7	20.28	2-heptadecanone	1.85±0.89	2.89±0.67	4.93±1.58	0.020	0.000
8	21.43	Dibutyl phthalate	1.68±0.20	1.54±0.21	1.77±0.66	0.065 [b]	0.718
9[c]	22.36	Hexadecyl acetate [a]	4.89±1.10	3.48±0.34	1.30±1.22	0.004	0.000
10	25.46	Hexadecanamide	1.19±0.96	6.46±2.83	5.53±3.47	0.001	0.005
11	28.48	Oleyl amide	7.02±5.13	9.64±2.90	15.86±5.72	0.230	0.005
12	31.34	Diisooctyl phthalate	5.48±0.81	5.40±0.47	5.75±1.89	0.809	0.703
13	33.42	Unidentified	0.86±0.61	2.11±1.09	3.23±2.85	0.014	0.039
14	34.78	Squalene	7.25±4.06	7.96±2.93	25.19±13.51	0.693	0.002 [b]

RF Sexually receptive female; *MM* Mate-searching male; *EF* Egg-holding female

[a] Compounds were verified with synthetic standard samples after separation with a non-polar column and a polar column; other components were identified tentatively by comparison with spectra listed in the NIST (Agilent Technologies 2002) mass spectral library and analogous data except for the unidentified compounds, whose mass spectra has low matching degree with those suggested by the MS library (NIST 2002)

[b] *P* values were tested by using Mann–Whitney U test; others were tested by using independent *T* test

[c] Relative abundances of these components in silk extract of sexually receptive females was significantly more than those in silk extracts of mate-searching males and egg-holding females

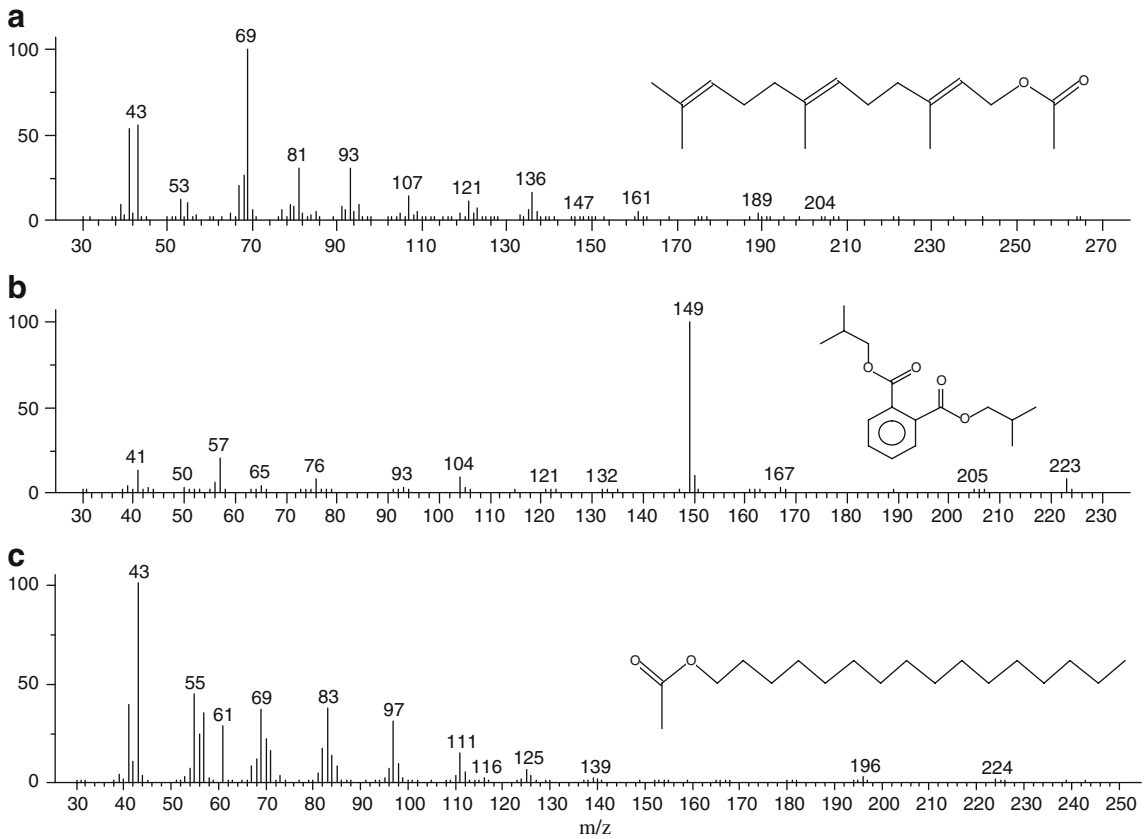

Fig. 4 Mass spectra of **a** compound 5, **b** compound 6, and **c** compound 9 of the silk sample. They were identified as (*E,E*)-farnesyl acetate, diisobutyl phthalate, and hexadecyl acetate, respectively, by comparing retention times and mass spectra with those of the authentic standards

Titer Analysis of the Putative Pheromone Components

The quantities of FA and HA on female webs (N=8) was determined by external regression analysis of concentration ranges for synthetic standards of these compounds. Sexually receptive females produce about 0.052 and 0.025 μg/web of FA and HA, respectively.

Biological Activity of the Potential Pheromones

Bioassay results showed that male *P. beijingensis* displayed no choice for a particular chamber in the trials involving the single compounds FA, DIBP, or HA (X_1^2=0.529, P>0.05; X_1^2=0.222, P>0.05; X_1^2=0.474, P>0.05, respectively; Fig. 5). However, when we combined the three compounds, the tertiary blend of FA, HA, and DIBP was attractive to selecting males (X_1^2=4.263, P<0.05; Fig. 5). The binary blend of FA and HA also showed significant attraction to males (X_1^2=4.000, P<0.05; Fig. 5), while the other two binary blends did not attract males (X_1^2=0.053, P>0.05; X_1^2=0.818, P>0.05) ; Fig. 5). Whereas FA and HA were required to attract males, DIBP was not, even if it occurred in a large amount in the silk. As the silk samples were collected from the cardboard boxes with a slice of plastic paper filmed in the inner wall, we cut down a small chip of the plastic paper (3×3 mm) and extracted it with dichloromethane. GC-MS detection result showed that the phthalate compounds including diisobutyl phthalate, dibutyl phthalate, and diisooctyl phthalate were presented in the extraction. Therefore, the phthalates must be contaminants introduced on the webs by the spiders coming in contact with the plastic.

Attractiveness of the binary blend of FA and HA in different concentrations was tested (Fig. 6). Male *P. beijingensis* significantly preferred the chamber containing FA and HA at concentrations of 0.01 and 0.1 μg/μl (X_1^2= 4.571, P<0.05; X_1^2=7.118, P<0.05, respectively; Fig. 6). Males showed no significant choice between the treatment chamber and control chamber in the trials involving the binary blend at concentration of 0.001 μg/μl and the highest concentration of 1 μg/μl (X_1^2=1.143, P>0.05; X_1^2=2.579, P>0.05; Fig. 6).

Most males displayed exploratory behavior (Table 1, Level 1) but no males displayed courtship signaling (Table 1, Levels 2 and 3) when presented with pheromone solutions on filter-paper on the webs made by egg-holding females. Likewise, only searching behavior, and no courtship signaling, was exhibited by males on the webs produced by sexually

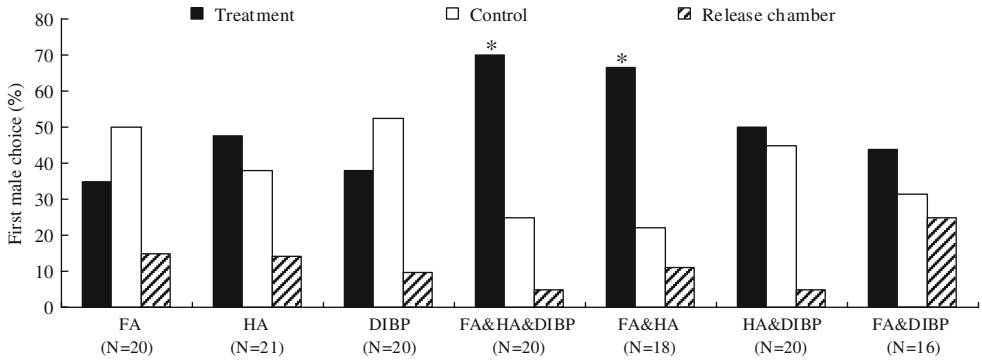

Fig. 5 Results of the attractiveness for male *Pholcus beijingensis* to single putative pheromone component and the blends. Trials were completed in the two-choice arena system (Fig. 1). Dosage of the chemicals applied in the treatments was equal to those obtained from one female web. 'Control' means equal quantity dichloromethane to corresponding applied chemicals. 'Release chamber' refers to males that within the 2 h observation period failed to leave the central chamber into which they had been introduced 1 h before the start of the trial. * indicates $P < 0.05$

receptive females. Male *P. beijingensis* initiated signaling only when a sexually receptive female was on the web.

Discussion

The sex pheromone of *P. beijingensis* consists of a 2:1 ratio of (*E,E*)-farnesyl acetate (FA) and hexadecyl acetate (HA), deposited on the web by sexually receptive females. By triggering male searching behavior, the pheromone plays an important role in guiding males to females' webs. Male *P. beijingensis* showed no attraction response to either FA or HA alone. Although both females and males of *P. beijingensis* can emit the pheromone, sexually receptive females release much more than males. FA is relatively abundant on the female's silk, averaging 0.052 μg per web, while HA averages 0.025 μg per web. Mixtures of FA and HA at or below 0.01 μg and 0.005 μg, respectively, failed to attract selecting males. Thus, male *P. beijingensis* have an 'olfactory detection threshold' for finding potential mates. Pheromone in a slightly higher concentration than that generally found on the web of a sexually receptive female attracted males and stimulated mate-searching behavior (Fig. 6). However, males were not attracted when the pheromone dosage was much higher than that of a sexually receptive female's web.

In other species of spiders, however, single pheromone compounds bound to the silk act as sex attractants (Papke et al. 2000, 2001; Tichy et al. 2001). The first reported spider sex pheromone, that of *Linyphia triangularis*, comprised two compounds, (*R*)-3-hydroxybutyric acid (HBA) and (*R*)-3-[(*R*)-3-hydroxybutyryloxy]-butyric acid (HBBA), and either compound alone could trigger the web reduction behavior of males. The dimer (HBBA) and the monomer (HBA) are relatively unstable compounds, such that HBBA slowly disintegrates into the monomer, HBA. Thus, HBBA

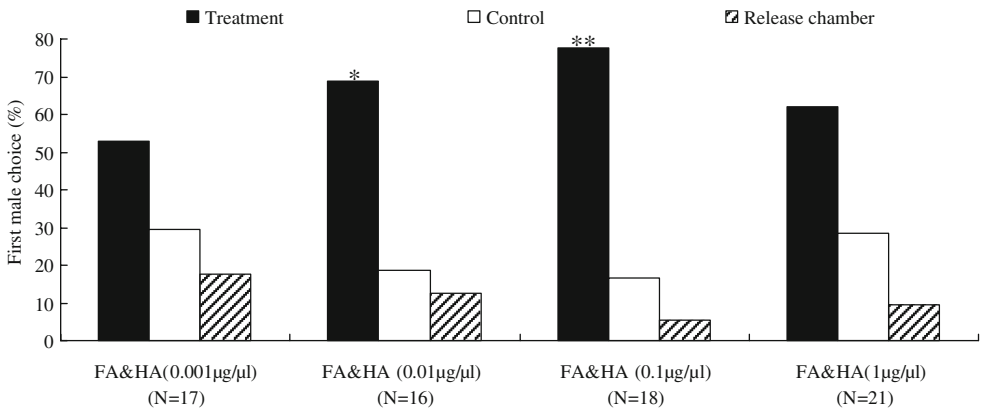

Fig. 6 Results of the attractiveness for male *Pholcus beijingensis* to four concentrations of the two-component blend and each component of the blend. Trials were completed in the two-choice arena system. FA is (*E,E*)-farnesyl acetate and HA is hexadecyl acetate. The category 'treatment' refers to one of the stepwise concentrations of the binary blend. 'Control' refers to equivalent dichloromethane to treatment. 'Release chamber' refers to males that within the 2 h observation period failed to leave the central arena into which they had been introduced 1 h prior to the start of the trial. * indicates $P < 0.05$. ** indicates $P < 0.01$

may be the precursor of HBA (Schulz and Toft 1993). (*R*)-HBA occurs in three closely related genera of spiders: *Linyphia*, *Microlinyphia*, and *Neriene* (Schulz and Toft 1993).

The major pheromone component of *P. beijingensis* (FA), was identified previously as a sex pheromone component of the click beetle, *Agriotes proximus* (Coleoptera: Elateridae) (Yatsynin et al. 1980, 1996). This compound also was reported from male Scandinavian bumblebees, *Bombus pratorum* (Bergman and Bergström 1997), the rock honeybee *Apis dorsata* (Blum et al. 2000), and the stingless bee *Melipona beecheii* (Cruz-López et al. 2005). Recently, FA was described as a sex pheromone component in the preputial gland of male Brandt's voles, *Lasiopodomys brandtii*, where it attracts females (Zhang et al. 2007b). HA also was described as a pheromone component in voles (Brinck and Hoffmeyer 1984; Welsh et al. 1988; Zhang et al. 2007a). Straight-chain acetates usually containing 12–18 carbons are common pheromone components in other insects (Roelofs 1995; Byers 2002).

Most insect sex pheromones are blends of chemicals that consist of a number of different components, and they function only when combined in a particular ratio (Wyatt 2003). Multicomponent pheromones also occur in vertebrates, such as goldfish, mice and other mammals; here too, individual components of the blend usually are inactive (Novotny et al. 1999; Sorensen and Stacey 1999). It is not uncommon that some pheromone components are used by several, distantly related species. The Asian elephant, *Elephas maximus*, shares its female sex pheromone with 140 species of moth (Rasmussen et al. 1996). Zhang et al. (2007b) reported that most of the castration-suppressed compounds in preputial gland secretions of male Brandt's voles were described previously as pheromone components of insects. The sex pheromone compounds from the spider *L. triangularis* (HBA and its dimer HBBA) also have been found in an ascomycete fungus, *Hypoxylon truncatum* (Quang et al. 2003). The coincidence of pheromone components among different species illustrates not only the ubiquity of pheromones, but also their biochemical convergence (Wyatt 2003).

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Response of *Mythimna unipuncta* Males to Components of the *Sesamia nonagrioides* Pheromone

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Abstract Several sympatric lepidopteran species feed on maize plants, and the different components of their species-specific female sex pheromones may play a role in attracting conspecifics and/or deter heterospecific males. In this study, we analyzed the content of *Mythimna unipuncta* pheromone glands and tested the response of males to components of their own pheromone blend and that of *Sesamia nonagrioides* in the wind tunnel. Whole pheromone glands, and lures where (Z)-9-hexadecenyl acetate, Z)-11-hexadecen-1-ol or (Z)-9-hexadecenyl acetate + (Z)-11-hexadecen-1-ol were added to the major component, (Z)-11-hexadecenyl acetate, elicited significantly higher responses by *M. unipuncta* males than lures with main component alone, although the levels varied with concentration. In the field a rather different outcome was observed, as the addition of other compounds found in the female pheromone gland did not improve trap catch over lures with only (*Z*)-11-hexadecenyl acetate. The addition of (*Z*)-11-hexadecenal, a compound of the *S. nonagrioides* pheromone, to (*Z*)-11-hexadecenyl acetate significantly reduced attraction of *M. unipuncta* males both in the wind tunnel and in the field, as well as the number of sympatric clover cutworm, *Discestra trifolii*, under field conditions. The addition of (Z)-9-hexadecenyl acetate, a minor component of the *M. unipuncta* pheromone blend, reduced the number of *S. nonagrioides* captured in field traps that were baited with the *S. nonagrioides* lure. The significance of such inhibition in the reproductive isolation of sympatric species that attack maize is discussed.

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Introduction

Pheromone mediated mating is common in insects, especially in nocturnal Lepidoptera. Species-specific pheromone blends not only facilitate mate location by conspecifics, but may also inhibit responses by heterospecific adults, serving as a reproductive isolating mechanism and reducing costly mating mistakes. However, such effects on heterospecific males have been reported, not only by closely related species with common components in the respective female sex pheromone blends, but also between taxonomically distant species with totally different pheromones. For example, Eizaguirre et al. (2007) found that the two principal components of the European corn borer (ECB), *Ostrinia nubilalis* pheromone inhibited the response of the *Sesamia nonagrioides* males to their conspecific pheromone. Similarly, when one component of the *S. nonagrioides* pheromone was added to the corn borer pheromone, it inhibited the response of ECB males. They hypothesized that this form of inhibition either could play a role in interspecific competition or be part of reproductive isolation mechanisms that involve a third species that is sympatric with the other two (Greenfield 2002).

In previous field studies (Albajes et al. 1988), traps baited with *S. nonagrioides* lures or virgin females attracted males of the sympatric noctuid, *Mythimna unipuncta*, a species that shares two common pheromone components with *S. nonagrioides* (Steck et al. 1980, 1982; Streng et al. 1985). Furthermore, the addition of ECB pheromone to the *S. nonagrioides* lure inhibited the responses of both *S.*

nonagrioides and *M. unipuncta* males (Eizaguirre et al. 2002). We, therefore, undertook both laboratory and field studies to determine the response of *M. unipuncta* males to lures that contain their own pheromone alone and when mixed different components of *S. nonagrioides* sex pheromone.

Methods and Materials

Mythimna unipuncta eggs were obtained from field collected females, and the resulting larvae were reared individually at 25°C, 16 L8 h D, 60% RH on an artificial diet (Shorey and Hale 1965). Pupae were sexed and then stored at 8°C until required. For the different experiments, male and female pupae were reared in two separate incubators at 21°C, 16 L8 h D, 60% RH, and the adults emerging each day were held in cages with a supply of 10% sucrose solution.

Identification of Female Sex Pheromone Samples of female *M. unipuncta* sex pheromone were obtained by gently extruding the glands and wiping the surfaces for 25 min with an SPME fibre (100 mm polydimethylsiloxane, Supelco), as described by Frerot et al. (1997). Each sample, taken from two to five individuals, was collected during the scotophase, using only females that were exhibiting calling behavior. The samples were analysed on a DB-Wax capillary column (30 m×0.25 mm ID×0.25 mm film thickness)(J & W Scientific, CA, USA) with an Agilent Technologies 6890 N gas chromatograph in splitless injection mode (45 s) coupled to an Agilent Technologies 5973 N mass spectrometer. The injector temperature was set at 250°C, and the GC oven temperature was programmed from 100°C (1 min) to 225°C at 5°C/min and then held at this temperature for 10 min. The SPME fibre was desorbed into the injector for 5 min. The pheromone components were subsequently identified by comparing retention times and fragmentation patterns with those of synthetic standards: [(*Z*)-11-hexadecenyl acetate, (*Z*)-11-hexadecen-1-ol, (*Z*)-11-hexadecenal, dodecyl acetate, (*Z*)-9-hexadecenyl acetate, and hexadecyl acetate] purchased from SEDQ SA (Barcelona, Spain). All were found to be >96% isomerically pure by GC analysis. The N-hexane used, purchased from Acros Organics (Geel, Belgium), was of pesticide residue grade.

Male Responses in a Wind Tunnel Assays were carried out in a laminar flow wind tunnel (Gemeno et al. 2006) at 21± 1°C, 50% RH and a wind velocity of 0.2 m/s during the 3–5th h of the scotophase (the peak response period, unpublished data). The tunnel was illuminated with a fluorescent red light resulting in a 1.8 and 1.5 lux intensity at the ceiling and floor, respectively.

Two series of assays were done by testing various combinations of the compounds identified in *M. unipuncta* female pheromone glands, and those from the *S. nonagrioides* pheromone blend. All compounds tested in the assays (see Table 1) were applied to cardboard female mimics, previously washed with hexane, suspended from the center of the ceiling at the upwind end of the tunnel. When the natural pheromone sources were tested, glands from two calling females were excised and immediately placed on the mimic to be tested. Individual males were released 1.4 m downwind of the pheromone source to be tested, and were observed for a maximum of 5 min, with the following behaviors being recorded: taking flight (TF), oriented upwind flight (ZZ), and contacting the source (CT). The proportion of males any given behavior was calculated by using only those individuals that completed the previous step and not the total number used in a given assay. Five to ten males were flown to any given source during an assay, with a total of 30–40 males tested to each source during the entire experiment. Males varied in age from 3–6 days-old, with each individual being tested only once.

Field Trials Trials were carried out in the Lleida region of Spain (utm-31n, *X*=291035 *Y*=4620587) from July 26 to October 11 in 2006 and July 18 to November 7 in 2007, using a randomised block design, with blocks being separated by >300 m. Econnex®-type universal moth traps, with dichlorvos to kill the captured insects, were hung on 1.5-m-high wooden stakes, placed 20 m apart within each block. The compounds tested (see Table 1) were applied to rubber septa (Sigma-Aldrich Química, S. A.) that had previously been washed with hexane for 1 h, and attached with wire from the ceiling of the trap. Lures were not replaced during the trapping period. The number of *M. unipuncta*, *S. nonagrioides*, *O. nubilalis*, and *Discestra. trifolii* (a noctuid that feeds on alfalfa and is usually caught in traps baited with *S. nonagrioides* pheromone) males were recorded weekly.

Statistical Analyses There were no differences in the proportion of males taking flight when exposed to the different lures, so only the percentages of males that exhibited oriented flight (ZZ) and contacted the lure (CT) were compared. Furthermore, as there was no effect of age on male response to any given lure, the behaviors of all males for any given treatment were pooled before analyses by using the normal approximation of the binomial tests.

Trap catch data were transformed (square root [x+0.5]) and then analyzed with a split-split-plot-like model (Gomez and Gomez 1984), and the means compared by using LSD. Statgraphics (1997) was used for all analyses.

Table 1 Composition of the lures (μg) used in flight (T) and field (F) experiments on *Mythimna unipuncta*

Nomenclature	(Z)-11-hexadecenyl acetate	(Z)-11-hexadecen-1-ol	(Z)-11-hexadecenal	Dodecyl acetate	(Z)-9-hexadecenyl acetate	hexadecyl acetate	Experiments
SePh	77	8	10	5	-	-	F,T
Pp	10	-	-	-	-	-	F,T
Pp + OH	100 (100)	5 (10)	-	.	-	-	F,T (T)
Pp + Ald	100 (100)	-	5 (1)	-	-	-	F,T, (T)
Pp + 12	100	-	-	5	-	-	F,T
Pp + Z9	100 (100)	-	-	-	1 (5,10)	-	F,T (T)
Pp + OH + Z9	100	5	-	-	1	-	F
Pp + Ald + Z9	100	-	5	-	1	-	F
Pp + 12 + Z9	100	-	-	5	1	-	F
SePh + Z9	77	8	10	5	1	-	F
SePh-Ald	77	8	-	5	-	-	T
Pp + 16	100	-	-	-	-	10,15	T
Z9	100						T
MyGl							T
SeGl							T
WL	0	0	0	0	0	0	F,T

Those in brackets lures only tested in the tunnel flight assays

MyGl 2 *M. unipuncta* female glands, *SeGl* 2 *S. nonagrioides* female glands, *WL* mock female without lure, *Pp* (*Z*)-11-hexadecenyl acetate, *OH* (*Z*)-11-hexadecen-1-ol, *12* dodecyl acetate, *Z9* (*Z*)-9-hexadecenyl acetate, *SePh* the commercial pheromone blend for *S. nonagrioides*, *SePh-Ald* the commercial pheromone blend for *S. nonagrioides* without (*Z*)-11-hexadecenal

Results

Five different components were found in the gland extracts (Fig. 1), that by comparison with the retention times and MS fragmentation patterns of synthetic standards, were identified in order of relative quantities as (*Z*)-11-hexadecenyl acetate (100%), hexadecyl acetate (15–20%), (*Z*)-11-hexadecen-1-ol (2–3%), (*Z*)-9-hexadecenyl acetate (1%), and hexadecenol (1%). The sixth peak (marked with * in Fig. 1) is probably an impurity, as its mass spectra did not match any typical pheromonal component of Lepidoptera, and according to the NBS75K mass spectra library (from NIST), the best match (83%) was for glycerine.

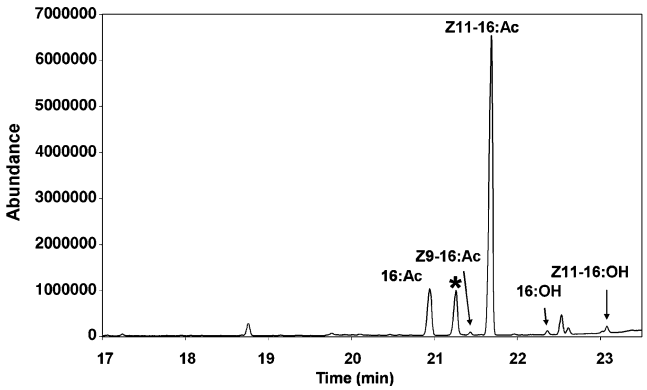

Fig. 1 Chromatogram obtained from an SPME wipe of 3 pheromone glands from 3- to 4-day-old calling *Mythimna unipuncta* females during the 4–5 h of the scotophase. The peak marked with an * is probably an impurity

The proportion of males taking flight was the same for all treatments (χ^2 test, $P=0.507$), so clearly none of the compounds tested in any of the wind tunnel assays inhibited flight behavior. Consequently, only the proportions of males that exhibited upwind flight and reached the source were considered.

In the first series of assays that tested the pheromone glands of both *S. nonagrioides* and *M. unipuncta* pheromone glands, as well as various combinations of synthetic blends, (*Z*)-11-hexadecenyl acetate + (*Z*)-11-hexadecenal, the *Sesamia* pheromone, (*Z*)-9-hexadecenyl acetate, or control mock females did not elicit any upwind flight by *M. unipuncta* males (data not shown). The highest responses of *M. unipuncta* males that oriented by flying upwind and reaching the source were observed with the conspecific female pheromone gland. The addition of either (*Z*)-9-hexadecenyl acetate or (*Z*)-11-hexadecen-1-ol to (*Z*)-11-hexadecenyl acetate resulted in a significantly higher proportions of males exhibiting upwind flight compared to the major pheromone component alone (Fig. 2), but only the presence of (*Z*)-9-hexadecenyl acetate resulted in a significant increase in the proportion contacting the source. Very few *M. unipuncta* males exhibited oriented flight, and none touched the source

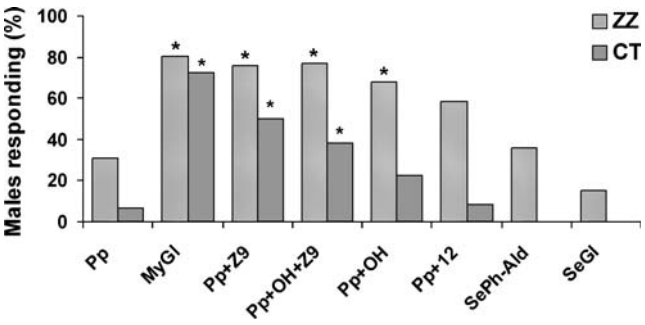

Fig. 2 Percent of *Mythimna unipuncta* males flying upwind in a zig-zag (ZZ) and contacting the source (CT) to different compounds embedded in a mock female in the wind tunnel. MyGl indicates two *M. unipuncta* female glands; Pp is 0.1 μg of the major *M. unipuncta* pheromone component (*Z*)-11-hexadecenyl acetate, while Pp+OH, 12, or Z9 indicate the addition (*Z*)-11-hexadecen-1-ol, dodecyl acetate or (*Z*)-9-hexadecenyl acetate (5% and 1%, respectively). SeGl indicates two *Sesamia nonagrioides* female glands while SePh-Ald indicates the commercial pheromone blend for *S. nonagrioides* without (*Z*)-11-hexadecenal;. Columns with * indicate significantly different response in comparison with the major component alone ($P<0.05$)

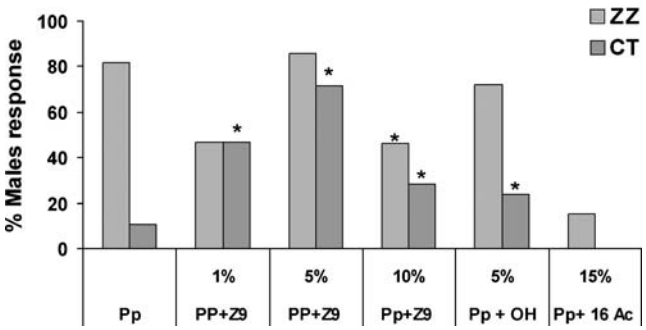

Fig. 3 Percent of *Mythimna unipuncta* males flying upwind in a zig-zag (ZZ) and contacting the source (CT) to different compounds embedded in a mock female in the wind tunnel. Pp is 0.1 μg of the major *M. unipuncta* pheromone component (*Z*)-11-hexadecenyl acetate, while Pp + Z9, OH, or16 indicate the addition of (*Z*)-9-hexadecyl acetate, (*Z*)-11-hexadecen-1-ol, or hexadecyl acetate to the major component, in the proportions shown. Columns with * indicate significantly different response in comparison with the major component alone ($P<0.05$)

when exposed to models with *S. nonagrioides* pheromone glands or to this species' synthetic blend, even when (*Z*)-11-hexadecenal was not included.

In the second set of assays, males exhibited no upwind flight when the major component, (*Z*)-11-hexadecenyl acetate, was combined with 10% of either (*Z*)-11-hexadecen-1-ol or hexadecyl acetate found in *M. unipuncta* pheromone glands, or with 1% or 5% (*Z*)-11-hexadecenal found only in the *S. nonnagrioides* pheromone blend (data not shown). However, for the other combinations, there were significant differences in both the proportions that exhibited upwind flight and contacted the source (Fig. 3). The proportion of males exhibiting upwind flight to the major component alone, was much higher than in the first series of assays (Fig. 2). The combinations of (*Z*)-11-hexadecenyl acetate with 1% and 5% (*Z*)-9-hexadecenyl acetate elicited the highest responses, although the addition of 10% (*Z*)-9-hexadecenyl acetate or 5% (*Z*)-11-hexadecen-1-ol still resulted in significantly more males reaching the source than with (*Z*)-11-hexadecenyl acetate alone. The addition of 15% hexadecenyl resulted in fewer males showing an upwind flight response, and none reached the source.

Field Trials In contrast to the wind tunnel results, the addition of different components identified from the pheromone gland of *M. unipuncta* did not increase trap catch, when compared to lures that contained just the major component, (*Z*)-11-hexadecenyl acetate. In fact, the addition of (*Z*)-11-hexadecen-1-ol resulted in significantly lower numbers of males being caught (Fig. 4). While the addition of (*Z*)-11-hexadecenal, one of the two components of the *S. nonagrioides* pheromone not found in the *M. unipuncta* gland, significantly reduced trap catches, the other, dodecyl acetate, had no effect (Fig. 4).

The pheromone blend identified by Sans et al. (1997) was the most attractive to *S. nonagrioides* males. Captures were low with (*Z*)-11-hexadecenyl acetate alone and while the addition of (*Z*)-11-hexadecenal did significantly increase the number of males captured, the addition of either (*Z*)-11-hexadecen-1-ol, (*Z*)-9-hexadecenyl acetate, or dodecyl acetate did not (Fig. 5). The addition of (*Z*)-9-hexadecenyl acetate, found only in the glands of *M.*

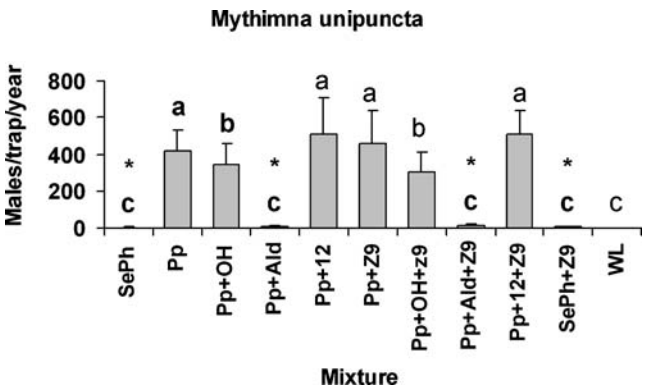

Fig. 4 Number of males (+S.E.) of *Mythimna unipuncta* caught per trap per year. All lures except WL contained (*Z*)-11-hexadecenyl acetate, the major pheromone component of *M. unipuncta* and *Sesamia nonagrioides*. Pp represents 100 μg of (*Z*)-11-hexadecenyl acetate, while Pp + OH, Ald or 12 indicate the addition of (*Z*)-11-hexadecen-1-ol, (*Z*)-11-hexadecenal, or dodecyl acetate (5 μg), respectively. Z9 indicates the addition of 1 μg of (*Z*)-9-hexadecenyl acetate, SePh is the complete pheromone of *S. nonagriodes* (Sans et al. 1997), and WL indicates empty traps. Catches in traps where the lures contained (*Z*)-11-hexadecenal are marked with an asterisk. Different letters on the top of bars indicate significant differences in the numbers of males captured

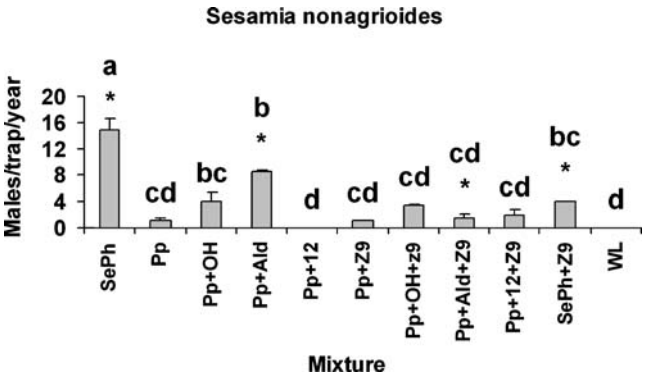

Fig. 5 Number of males (+S.E.) of *Sesamia nonagrioides* caught per trap per year. All lures except WL contained (*Z*)-11-hexadecenyl acetate, the major pheromone component of *Mythimna unipuncta* and *S. nonagrioides*. Pp represents 100 µg of (*Z*)-11-hexadecenyl acetate, while Pp + OH, Ald or 12 indicate the addition of (*Z*)-11-hexadecen-1-ol, (*Z*)-11-hexadecenal, or dodecyl acetate (5 µg), respectively. Z9 indicates the addition of 1 µg of (*Z*)-9-hexadecenyl acetate, SePh is the complete pheromone of *S. nonagriodes* (Sans et al. 1997), and WL indicates empty traps. Catches in traps where the lures contained (*Z*)-11-hexadecenal are marked with an asterisk. Different letters on the top of bars indicate significant differences in the number of males captured

unipuncta, to the complete *S. nonagrioides* blend resulted in a significantly lower trap catch compared with the complete blend alone (Fig. 5).

The lures that contained various components of *M. unipuncta* pheromone were all equally attractive to *D. trifolii* males (Fig. 6) but, as observed in *M. unipuncta* (Fig. 4), the addition of (*Z*)-11-hexadecenal caused a decrease in the number of males caught.

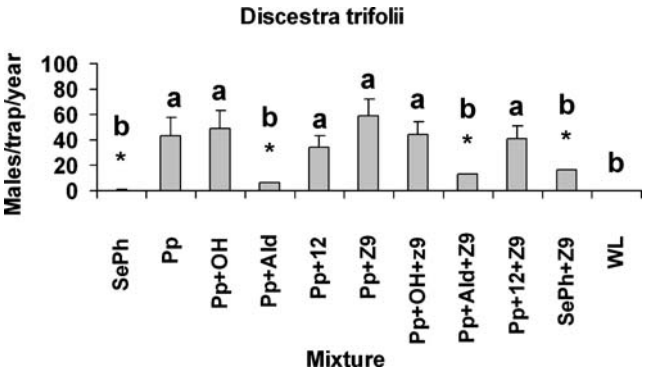

Fig. 6 Number of males (+ S.E.) of *Discestra trifolii* caught per trap per year. All lures except WL contained (*Z*)-11-hexadecenyl acetate, the major pheromone component of *Mythimna unipuncta* and *Sesamia nonagrioides*. Pp represents 100 µg of (*Z*)-11-hexadecenyl acetate, while Pp + OH, Ald, or 12 indicate the addition of (*Z*)-11-hexadecen-1-ol, (*Z*)-11-hexadecenal, or dodecyl acetate (5 µg), respectively. Z9 indicates the addition of 1 µg of (*Z*)-9-hexadecenyl acetate. SePh is the complete pheromone of *S. nonagriodes* (Sans et al. 1997), and WL indicates empty traps. Catches in traps where the lures contained (*Z*)-11-hexadecenal are marked with an asterisk. Different letters on the top of bars indicate significant differences in the number of males captured

Discussion

To date, five compounds have been identified from the pheromone glands of *M. unipuncta* (McDonough et al. 1980; Steck et al. 1980, 1982; Farine et al. 1981; Kamm et al. 1982). However, based on a number of different studies, only the major component (Z)-11-hexadecenyl acetate and (Z)-11-hexadecen-1-ol appear to play a major role in attracting males to lures. Even the role of (Z)-11-hexadecen-1-ol is somewhat unclear; while its addition to the main component did increase the number of males contacting the source in our wind tunnel experiments and has increased trap catches in some field studies (Steck et al. 1980; Turgeon et al. 1983), in others it did not (Farine et al. 1981; Kamm et al. 1982, this study). As noted by Turgeon et al. (1983), there are a number of non-mutually exclusive reasons for this apparent contradiction, including trap type, the height of the traps, the cropping system, and the position of the lure. A number of biotic and abiotic conditions may impact pheromone-mediated mating behavior (McNeil 1991) so the outcome may be affected by differences in the duration of trapping period, the local population densities, the frequency with which traps are emptied, and how the data are analyzed. For example, Turgeon et al. (1983) compared daily trap catch data, while in this study we emptied traps weekly and considered total numbers/trap for the entire season.

The majority of compounds found in the pheromone blend of a species are believed to play an intraspecific role in the long and/or short range responses of individuals searching for conspecific partners (Haynes and Birch 1985; David and Birch 1989). However, taken together, the results of various experiments conducted to date on the armyworm would support the idea put forward by Kamm et al. (1982) stating " it may be that *M. unipuncta* only utilizes (Z)-11-hexadecenyl acetate for long range attractancy". (Z)-11-Hexadecenyl acetate is a pheromone component in a number of Lepidoptera (McDonough et al. 1980; Sreng et al. 1985; Batiste-Pereira et al. 2006), many of which are sympatric. Thus, if specific components found in the pheromone gland of *M. unipuncta* do not serve in intraspecific mate location they could serve interspecifically to reduce the probability of heterspecific matings between sympatric species (Farine et al. 1981).

Clearly, the addition of (Z)-9-hexadecenyl acetate, a minor component found in the pheromone blend of *M. unipuncta* significantly reduced the effectiveness of the *S. nonagrioides* lure (Fig. 5), while not affecting *M. unipuncta* responses when added to (Z)-11-hexadecenyl acetate (Fig. 4). The addition of (Z)-11-hexadecenal, a minor component found in *S. nonagrioides* female pheromone glands and that is an essential component of the most effective *S. nonagrioides* lure (Sans et al. 1997), resulted in

a significant decline in the attractiveness of (Z)-11-hexadecenyl acetate for *M. unipuncta* (Fig. 4) and *D. trifolii* (Fig. 6). These results are in contrast to previous findings that traps baited with *S. nonagrioides* females captured *M. unipuncta* males (Albajes et al. 1988), and further field research is needed to determine under what ecological conditions such attraction might occur.

Interestingly, (Z)-9-tetradecenyl acetate, a pheromone component of *S. cretica* that inhibits *S. nonagrioides* males (Germinara et al. 2007), has also been reported to significantly reduce the responsiveness of *M. unipuncta* males to (Z)-11-hexadecenyl acetate, although the reported concentrations required to do so varies from >5 (Steck et al. 1980) to >20% (Kamm et al. 1982). However, *M. unipuncta* does not share any pheromone components with *O. nubilalis*, so the hypothesis that pheromone cross-inhibition between *S. nonagrioides* and *O. nubilalis* evolved to avoid attraction to a third sympatric species (Eizaguirre et al. 2007), would not involve the common armyworm.

Thus, there is a great deal of research required to determine to what extent the different acetates, and other pheromone components, serve in intra-specific attraction and/or inter-specific reproductive isolation in a number of sympatric species found in maize ecosystems. This information would not only provide basic information on the role of sex pheromones in mate choice, but would be of considerable importance in the use of pheromones in pest management programs that target a number of different species attacking the same host crops.

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Synergistic Interactions Between Cry1Ac and Natural Cotton Defenses Limit Survival of Cry1Ac-resistant *Helicoverpa Zea* (Lepidoptera: Noctuidae) on *Bt* Cotton

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Abstract Larvae of the bollworm *Helicoverpa zea* (Boddie) show some tolerance to *Bacillus thuringiensis* (*Bt*) Cry1Ac, and can survive on Cry1Ac-expressing *Bt* cotton, which should increase resistance development concerns. However, field-evolved resistance has not yet been observed. In a previous study, a population of *H. zea* was selected for stable resistance to Cry1Ac toxin. In the present study, we determined in laboratory bioassays if larvae of the Cry1Ac toxin-resistant *H. zea* population show higher survival rates on field-cultivated *Bt* cotton squares (= flower buds) collected prebloom—bloom than susceptible *H. zea*. Our results show that Cry1Ac toxin-resistant *H. zea* cannot complete larval development on Cry1Ac-expressing *Bt* cotton, despite being more than 150-fold resistant to Cry1Ac toxin and able to survive until pupation on Cry1Ac toxin concentrations greater than present in *Bt* cotton squares. Since mortality observed for Cry1Ac-resistant *H. zea* on *Bt* cotton was higher than expected, we investigated whether Cry1Ac interacts with gossypol and or other compounds offered with cotton powder in artificial diet. Diet incorporation bioassays were conducted with Cry1Ac toxin alone, and with gossypol and 4% cotton powder in the presence and absence of Cry1Ac. Cry1Ac toxin was significantly more lethal to susceptible *H. zea* than to resistant *H. zea*, but no difference in susceptibility to gossypol was observed between strains. However, combinations of Cry1Ac with gossypol or cotton powder were synergistic against resistant, but not against susceptible *H. zea*. Gossypol concentrations in individual larvae showed no significant differences between insect strains, or between larvae fed gossypol alone vs. those fed gossypol plus Cry1Ac. These results may help explain the inability of Cry1Ac-resistant *H. zea* to complete development on *Bt* cotton, and the absence of field-evolved resistance to *Bt* cotton by this pest.

Keywords *Helicoverpa zea* · Cry1Ac resistance · *Bt crops* · Gossypol · Fitness costs · Lepidoptera · Noctuidae

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Introduction

Bollworm, *Helicoverpa zea* (Boddie), has a naturally high tolerance to the *Bacillus thuringiensis* (*Bt*)-derived Cry1Ac protein compared to other target pests of *Bt* cotton in the US such as tobacco budworm, *Heliothis virescens* (F.), and pink bollworm, *Pectinophora gossypiella* (Saunders) (MacIntosh et al. 1990b; Sivasupramaniam et al. 2008). This relatively high tolerance, coupled with preferential feeding on tissues that express lower levels of *Bt* protein (Brickle et al. 2001), and toxin attenuation due to abiotic stress and plant phenology (e.g., postbloom), can result in complete larval development on Cry1Ac cotton, especially when insect populations are large (Jackson et al. 2004a). Additionally, *H. zea* is exposed to the *Bt* Cry1Ab protein in Cry1Ab-expressing corn, which is similar in structure and function to

Cry1Ac (Crickmore et al. 1998). This relatively high selection pressure increases the likelihood of resistance evolution (Gould 1998), and it was thus not surprising that early mathematical models predicted resistance development to *Bt* cotton in *H. zea* within 3–7 yr (Harris 1991; Roush 1997). Furthermore, the capacity for *H. zea* to develop resistance to Cry1Ac has been demonstrated in laboratory-selected strains (Luttrell et al. 1999; Jackson et al. 2004b; Anilkumar et al. 2008a). However, contrary to these results and predictions, field-evolved resistance has not occurred in *H. zea* even after 12 yr of intensive commercial use of *Bt* cotton in the USA (Ali et al. 2008; Moar et al. 2008).

Although numerous Cry1Ac-resistant strains of *H. virescens* (Gould et al. 1992, 1995), *P. gossypiella* (Liu et al. 1999; Tabashnik et al. 2000), and *Helicoverpa armigera* (Hubner) (Akhurst et al. 2003) have been developed in the laboratory, very few strains have been able to pupate and produce fertile adults on *Bt* cotton (Tabashnik et al. 2003; Bird and Akhurst 2004). Of those lepidopteran strains that can develop to fertile adults on *Bt* cotton, there is little correlation between the relative susceptibility of these strains to Cry1Ac, the level of Cry1Ac resistance in these resistant strains, and the relative survivorship of these resistant strains on *Bt* cotton (Liu et al. 1999; Akhurst et al. 2003; Tabashnik et al. 2003; Bird and Akhurst 2004). Possible explanations for this low correlation include interactions between *Bt* proteins and secondary plant metabolites (Carrière et al. 2004), use of a form of *Bt* protein for resistance selection not exclusively found in *Bt* cotton (Liu et al. 1999; Tabashnik et al. 2000; Akhurst et al. 2003; Henneberry and Jech 2007; Anilkumar et al. 2008a), increased consumption of plant tissues compared to consumption of artificial diet (Woods 1999), loss of genes or a reduction in expression of compounds necessary to survive on cotton during laboratory rearing and *Bt* resistance selection, or fitness costs associated with *Bt* resistance (Tabashnik et al. 2003; Gassmann et al. 2009).

Anilkumar et al. (2008a) reported a population of *H. zea* that was selected for stable and moderately high levels of resistance to the Cry1Ac toxin in the laboratory. Because *H. zea* is relatively tolerant to Cry1Ac, and arguably the most polyphagous of all target lepidopteran pests of *Bt* cotton that express Cry1Ac in the US, research was conducted to determine if larvae of the Cry1Ac toxin-resistant *H. zea* population show higher survival rates or can at least survive to a greater instar than susceptible *H. zea* on field-cultivated *Bt* cotton squares (= flower buds) collected during prebloom—bloom stage. Furthermore, this study explored the interaction of Cry1Ac with gossypol and cotton powder in artificial diet to help explain the higher than expected mortality observed for Cry1Ac-resistant *H. zea* on *Bt* cotton.

Methods and Materials

Insect Strains A laboratory susceptible colony of *Helicoverpa zea* (SC) was established in September 2004 from a laboratory colony from Monsanto (Union City, TN, USA). A resistant strain (AR) was the product of selecting SC for resistance by exposing individual neonates to an artificial diet containing up to 500 µg *Bt* Cry1Ac toxin/g diet for 25 generations (Anilkumar et al. 2008a). Seven d after exposure to the Cry1Ac toxin, surviving molted larvae were transferred to untreated diet and reared until pupation (Ali et al. 2006; Anilkumar et al. 2008a; Sivasupramaniam et al. 2008). Resistance to Cry1Ac toxin was assessed at selected generations (Anilkumar et al. 2008a), and the AR strain was >150-fold resistant (based on artificial diet bioassays) when survivorship bioassays on *Bt* and non-*Bt* cotton squares were initiated.

In order to avoid complete loss of the AR strain due to fitness costs associated with Cry1Ac resistance selection and rearing in the laboratory for 26 generations (Anilkumar et al. 2008b), AR was crossed with a new Monsanto susceptible strain (from Union City, TN), SC1, resulting in a strain designated AR1. SC1 had higher LC_{50} (31.25 µg Cry1Ac toxin/g diet) values than SC (8.89–15 µg Cry1Ac toxin /g diet; Anilkumar et al. 2008a). Although both reciprocal crosses were attempted, only AR[♀] X SC1[♂] yielded a viable F_1 population due to mating costs associated with AR males (Anilkumar et al. 2008b). Because Cry1Ac resistance in *H. zea* is inherited as a co-dominant character (Burd et al. 2003; Anilkumar et al. 2008b), high levels of resistance (resistance ratio >50-fold compared to SC1) were observed in the F_1 generation (data not shown) confirming that AR1 was resistant to Cry1Ac toxin. AR1 was selected at the regular selection concentration of Cry1Ac (500 µg Cry1Ac toxin/g diet) for two generations (Anilkumar et al. 2008a).

Cry1Ac Toxin An *E. coli* strain expressing Cry1Ac protoxin from *B. thuringiensis* subsp. *kurstaki* strain HD-1 (provided by L. Masson, Biotechnology Research Institute, National Research Council, Montreal, Canada) was cultured, and the activated toxin prepared as indicated elsewhere (Pusztai-Carey et al. 1994; Moar et al. 1995b).

Lyophilized Leaf Tissue Powder Studies Lyophilized cotton (*Gossypium hirsutum*, C312) leaf tissue powder was supplied by Monsanto (St. Louis, MO, USA).

Cotton Plants Bt cotton, *Gossypium hirsutum* (DPL555) and the near isogenic non-*Bt* cotton (N*Bt*) (DPL491) were planted at the Prattville Agricultural Research Unit, Alabama Agricultural Research Station, Prattville, AL, U. S.A.. Planting dates for *Bt*- and N*Bt*-cotton were April 23rd

and 24th, 2007, respectively. Cotton plants were cultivated as per typical practices. N*Bt*-cotton plants were treated with imidacloprid at 1 oz/A on both July 13 and July 24, 2007 for aphid control, and tissues were used 5 d after treatment for bioassays. Cotton plants were >85 d old (prebloom-bloom) when squares were harvested. Pin-head to midpoint-stage squares (7–14 d old) were collected, transported to the laboratory, and stored at 4–7°C until needed, up to a maximum of 13 d.

Cry1Ac Protein Quantification in Plants Beginning on the day of field collection, and on days when cotton squares were removed from refrigeration and used in bioassays, a random sample of 10 *Bt* squares was placed at −80°C. After all bioassays were completed, all −80°C samples were shipped to Monsanto (St. Louis, MO, USA) for Cry1Ac protein quantification. Square tissues were lyophilized, and Cry1Ac expression was determined using ELISA and compared against a positive *Bt* cotton standard as described in Greenplate (1999) and Sivasupramaniam et al. (2008). Three replicate assays were conducted for each sample.

Bioassay: H. zea Survival and Development on Cotton Squares Moist cotton balls were immersed in distilled water, squeezed to remove excess water, and placed in petri dishes (35×10 mm) to reduce cotton square desiccation. Individual neonates from both *H. zea* strains were placed on the outside of square bracts on one square in each petri dish, and squares were changed every 3 d (replacing larvae on bracts) until the end of the experiments. For both *Bt* and N*Bt*-cotton tests, larvae and squares were transferred to 30 ml cups that contained three to five squares when larvae reached 4th instar, and rearing was continued until pupation. Larval mortality and stadia were recorded beginning on the 4th d, and subsequently at 3 d intervals. Larval weights were recorded after 7 d, and tests were continued until survivors reached pupation. Bioassay trays were incubated at 27±1°C, RH 50%, and a photoperiod of 14:10 (L:D) h. Thirty larvae from each strain were tested in each replication. Experiments were repeated three times.

Bioassays: H. zea Survival and Development on Differently Treated Food General Gossypol and/or Cry1Ac toxin were added when the diet temperature was <60°C, and the diet was mixed thoroughly. Diets were filled into 128-well bioassay trays (CD-International, Pitman, NJ, USA) at about 1 g per well. Neonate larvae of strains AR1 and SC1 were transferred individually into each well and covered with ventilated covers. Bioassay trays were incubated at 27±1°C, RH 50%, and a photoperiod of 14:10 (L:D) h. Larval mortality and stadia were recorded beginning on the 4th d, and subsequently at 3 d intervals, up to 25 d. IC_{50} values (μg Cry1Ac/g diet) were determined as those inhibitory concentrations at which half of the larvae failed to molt to the 3rd instar after 7 d. Larval weights were recorded after 7 d, and tests were continued until survivors reached pupation. EC_{50} or EC_{90} (μg Cry1Ac/g diet) were determined as those effective concentrations at which larval weight was reduced by 50% or 90% compared to larvae fed on an untreated control diet (Jalali et al. 2004). Thirty-two larvae were tested for each treatment. Three replicates were conducted for the diet with Cry1Ac and the diet with Cry1Ac plus gossypol; six replicates were run for the diet with Cry1Ac plus lyophilized cotton powder.

(a) *Artficial diet with Cry1Ac*. Larvae were exposed to a level of Cry1Ac toxin four to six times greater than detected in fresh *Bt* cotton squares by ELISA, i.e., 15 μg Cry1Ac toxin/g diet.
(b) *Artficial diet with Cry1Ac and gossypol*. Larvae were exposed to diet containing 3.75 μg, 7.5 μg, or 15 μg Cry1Ac/g diet, or 0.0375%, 0.075%, or 0.15% gossypol (95% in acetic acid crystals, Sigma, St. Louis, MO, USA), and a 1:1 combination of Cry1Ac and gossypol (at respective dilutions from the maximum concentration used). For control, larvae were kept on a diet without Cry1Ac and gossypol additions. Because gossypol was dissolved in 1.0 % dimethylsulfoxide (DMSO), all diets contained a final concentration of 1.0% DMSO.
(c) *Artificial diet with Lyophilized cotton powder and Cry1Ac*. Larvae were exposed to 4% cotton powder, 15 μg Cry1Ac/g diet, and their 1:1 combinations in two generations.

Quantification of Gossypol Uptake by Larvae Five larvae from each replication that survived after 7 d in bioassays that contained gossypol (gossypol alone, gossypol plus Cry1Ac) or cotton powder (cotton powder alone, cotton powder plus Cry1Ac) were weighed and placed individually into 30 ml plastic cups that contained no diet for 10–12 h to allow for purging of gut contents. Insects were transferred individually to a microcentrifuge tube and frozen at −80°C. Gossypol content per insect was determined as described by Orth et al. (2007).

Data Analysis

Effect of Storage on Cry1Ac Stability in Plants Plant age at sampling was considered a fixed classification effect, and duration of storage as a fixed effect covariate nested with age to analyze the effect of storage on Cry1Ac stability. The sole random effect in the model was replicate(age), and Proc Mixed predicted a separate intercept for each age class with the no intercept option (SAS Institute 2003).

Larval Feeding upon Cotton Squares Larval mortality was arcsine square root transformed and analyzed by repeated measures ANOVA with SAS Proc GLM using the normal distribution function. The effect of diet on larval development (instars 3 and 4) was modeled with SAS Proc GLIMMIX with a binomial distribution function and the logit link function. Strain, treatment, and their interactions were fixed effects and replicate the sole random effect. The degrees of freedom for *t*-tests and confidence intervals were calculated as the number of group means minus the number of fitted parameters (Schabenberger and Pierce 2004).

Larval Feeding upon Diet with Cry1Ac Larval mortality was arcsine square root transformed and analyzed by repeated measures ANOVA with SAS Proc GLM using the normal distribution function.

Larval Feeding upon Diet with Gossypol and Cry1Ac Mortality of larvae that feed upon these diets was modeled by logistic regression with SAS Proc NLmixed using the binomial distribution function. The CONTRAST statement was used to evaluate the statistical significance of toxin differences within strains and ESTIMATE to calculate IC_{50} values (see above) plus associated 95% confidence intervals, as well as the contrasts between strains. For larval weight data, replicate x strain interaction means in response to toxin rate were modeled with SAS Proc NLmixed by using an exponential decay model with a lower asymptotic limit and a normal distribution function. EC_{50} and EC_{90} values were estimated from this analysis (see above). Interactions of effects Cry1Ac and gossypol on larvae were evaluated as described by Salama et al. (1984). Differences in observed mortality and theoretical mortality for the mixture of Cry1Ac and gossypol were analyzed with χ^2 tests. Interaction was considered 1) synergistic, if observed mortality was more than expected mortality coupled with significant chi square values, 2) additive, if observed mortality was more than expected mortality coupled with non significant chi square values, 3) antagonistic, if observed mortality was less than expected mortality coupled with significant chi square values.

Larvae Feeding upon Diet with Lyophilized Leaf Tissue Powder and Cry1Ac Larval mortality was modeled with SAS Proc GLIMMIX with a normal distribution function. The residual variance was modeled by using the group option to account for heterogeneous variances among treatments. Larval weight and gossypol concentration/mg larva were modeled using the same procedure but with lognormal distribution function, which was necessary as residuals under the normal assumption were extremely right-skewed. Treatment, strain, and their interaction were treated as fixed effects. However, random effects were different for each of parameter. For larval weight, generation, replicate (generation), and their interactions with fixed effects. For larval mortality, generation and replicate (generation) were considered to be random effects. Least squares diet × strain interaction means were calculated. The slicediff (for larval weight and mortality), pdiff (gossypol concentration/larvae), and simulation options were employed to assess differences among strains and diets while controlling the Type I error rate. Analysis of synergism between Cry1Ac and cotton powder was evaluated as explained above.

Quantification of Gossypol in Larvae Since the distribution of gossypol concentration per larva was right skewed, data were analyzed with a generalized linear models framework that utilized SAS Proc GLIMMIX. The lognormal distribution function resulted in a symmetrical distribution of residuals. Strain and toxin were fixed effects class variables, and toxin rate was treated as a fixed effects covariate. Differences among toxin x strain combinations then were predicted at toxin rates 375, 750, and 1,500 using the AT option of the LSmeans statement with the simulation adjustment to control the Type I error rate.

Results

Cry1Ac Expression in Cotton Squares The concentration of Cry1Ac in our field-collected *Bt* cotton squares (flower buds) was not significantly different from levels of Cry1Ac found in Monsanto's positive control, DP50 Bollgard® squares used to determine if commercially grown Bollgard® is producing acceptable levels of Cry1Ac (Fig. 1). Cry1Ac expression was reduced (F_5=248.75, P< 0.001) by 45% from 86 d (July 18) to 124 d (August 25) after planting; and storage at 4–7°C did not affect the stability of the Cry1Ac for all samples except the July 18th samples (P=0.049).

Survivorship of Helicoverpa zea Larvae of AR and SC Strains on Bt Cotton and NBt Cotton Only tissue type (*Bt* and N*Bt*) ($F_{1,8}$=140.02, P<0.001), but not strains ($F_{1,8}$= 2.65, P=0.143) or their interactions ($F_{1,8}$=4.48, P=0.067) had a significant effect on cumulative mortality at 31 d (Fig. 2a). Mean (± SE) cumulative mortality on *Bt* cotton for SC larvae at 4 d and 7 d was 60.00 (±8.33) and 76.67 (±6.01) percent, respectively, and was significantly (P<0.05) higher compared to AR larvae (34.45±1.92 and 54.45 ± 1.93% at 4 d and 7 d, respectively). No significant differences were observed in cumulative mortality on 10 d, 13 d, and 16 d between AR (70.00±7.26, 77.78±6.94, and 81.11±7.51%) and SC larvae (86.67±4.41, 88.89±4.19, and 95.56±2.55%) on *Bt* squares. All SC larvae on *Bt* squares were dead by

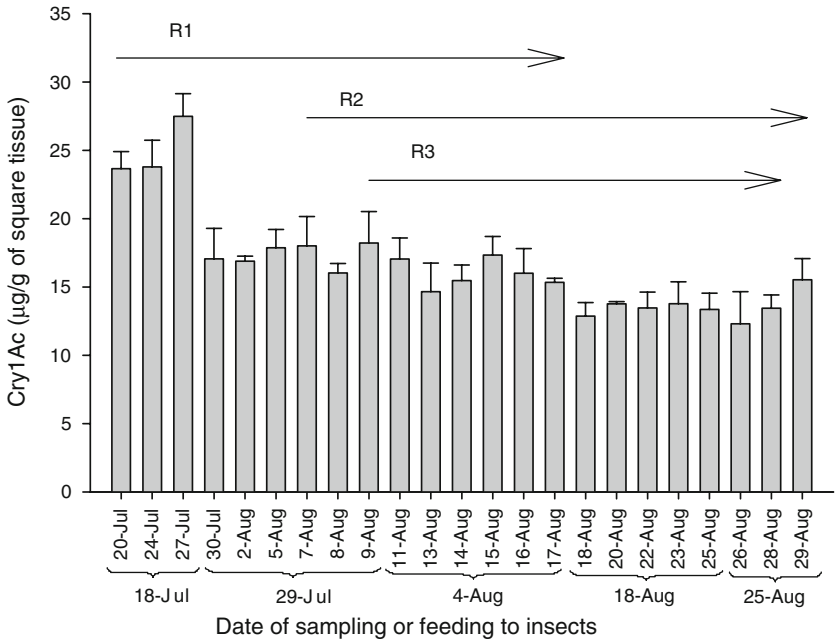

Fig. 1 Quantity of Cry1Ac protein (µg Cry1Ac/g lyophilized tissue) expressed in *Bt* cotton squares measured using ELISA. R1-3 = replicates 1–3. *Squares* were harvested July 18–Aug 25, and *squares* were refrigerated at 4–7°C until fed to *Helicoverpa zea* from July 20–Aug. 29. Means and SD of three replicates are given

19 d compared to 84.45±6.74% mortality for AR larvae, and these differences were significant ($P<0.05$). Both AR and SC larvae survived equally well on N*Bt* cotton; neither duration nor total survivorship varied between strains.

Larval weight after 7 d was different when comparing their food (*Bt* and N*Bt* cotton tissues ($P<0.001$), but not when comparing AR and SC strains ($P=0.406$): Neither was the interaction of food tissue and strain significant ($P=0.125$) (Table 1). When considering larval mortalitiy, tissue ($P<0.001$) and its interaction ($P=0.028$) with strain, but not the strain alone ($P=0.099$) had an effect on the number of larvae reaching 3rd instar. The proportion of larvae reaching 3rd instar differed significantly between AR and SC on *Bt*, but not on non-*Bt* tissues, and only AR-larvae reached 4th instar on *Bt* cotton squares.

Effect of Cry1Ac in Artificial Diet All factors such as strains ($F_{1,8}=22.32$, $P=0.002$), type of diet (Cry1Ac treated or untreated) ($F_{1,8}=80.20$, $P<0.001$), and their interactions ($F_{1,8}=40.56$, $P<0.001$) had effects on cumulative mortality after 25 d (Fig. 2b). For within subject effects, only duration of exposure ($F_{7,2}=49.92$, $P=0.020$) and its interaction with type of diet ($F_{7,2}=34.57$, $P=0.028$) had an effect on mortality over time. However, no differences were observed for interaction of duration of exposure with strains ($F_{7,2}=5.12$, $P=0.173$) nor for their combined interaction with type of diet ($F_{7,2}=11.18$, $P=0.085$). Larvae of SC1 and AR1 strains performed similarly on untreated diet resulting in 10–17% mortality after 25 d. SC1-larvae feeding on 15 µg Cry1Ac toxin/g diet had 92% mortality after 25 d, whereas AR1-larvae had only 45% mortality feeding on 15 µg

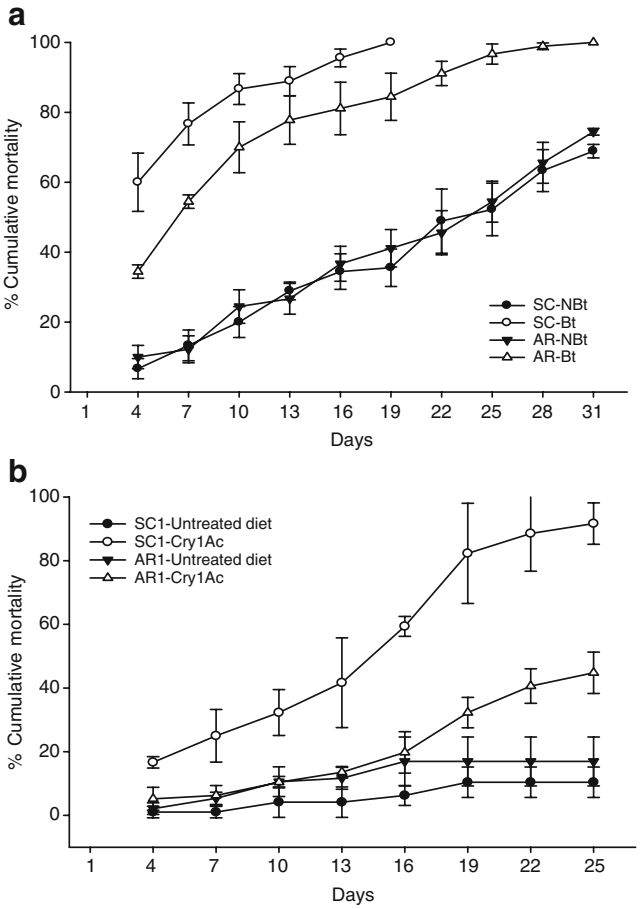

Fig. 2 Cumulative % mortality of susceptible (SC) and Cry1Ac-resistant (AR) *Helicoverpa zea* on **a** *Bt* (DPL-555) and N*Bt* (DPL-491) cotton squares and **b** untreated diet and diet treated with 15 µg Cry1Ac/g diet. Means and SD of three replicates are given

Table 1 Performance of Cry1Ac-resistant (AR) and susceptible (SC) *Helicoverpa zea* on *Bt* (DPL-555) and N*Bt* (DPL-491) cotton squares

Square	Strain	Larval mass at 7d (mg)	Proportions at the end of the experiment (31d)	
			3rd instars	4th instars
Bt	AR	0.93 (0.69–1.25)[a]	0.19 (0.07–0.42)	0.06 (0.02–0.15)
	SC	0.66 (0.45–0.96)	0.05 (0.02–0.16)	0.00
N*Bt*	AR	4.11 (3.16–5.35)	0.76 (0.51–0.91)	0.63 (0.51–0.74)
	SC	4.57 (3.51–5.95)	0.81 (0.58–0.93)	0.66 (0.53–0.76)
F-Test				
Strain		F_1=0.73, P=0.406	F_1=3.49, P=0.099	F_1=0.00, P=0.954
Tissue		F_1=155.74, P<0.001	F_1=108.0, P<0.001	F_1=0.00, P=0.970
Strain* Tissue		F_1=2.66, P=0.125	F_1=7.20, P=0.028	F_1=0.00, P=0.969

The test was conducted in three replicates of 30 insects each

[a] Values in the parenthesis are 95% confidence intervals

Cry1Ac toxin/g diet after 25 d. After 25 d, only 8.33% SC1-larvae reached pupation as compared to 55% pupation in AR1. For both strains, the largest increase in mortality occurred between 16–19 d. At this time mortality of AR1-larvae feeding upon Cry1Ac toxin increased significantly compared to the untreated control.

Effect of Cry1Ac and Gossypol in Artificial Diet IC_{50} values (i.e., Cry1Ac toxin concentration at which 50% of larvae fail to molt to third instar) were significantly different between AR1 (17.56) and SC1 (7.07) larvae. With the addition of gossypol to Cry1Ac toxin, however, the IC_{50} value for AR1-larvae (10.04) decreased significantly and was not significantly different (P=0.05) from SC1-larvae on Cry1Ac alone (Table 2). The IC_{50} value for SC1-larvae with the Cry1Ac plus gossypol combination did not significantly decrease (P=0.05) compared to Cry1Ac alone (Table 2). EC_{50} values (i.e., Cry1Ac toxin concentration at which larval weight was reduced by 50%) for AR1 (1,172) and SC1-larvae (1,204) were similar for gossypol, and were significantly higher than the values for Cry1Ac toxin (AR1=0.99, SC1=0.97) and their mixtures (AR1=1.35, SC1=0.94) (Table 3). Gossypol concentration in larvae did not differ significantly between strains at any concentration (375 µg/g diet, 750 µg/g diet and 1,500 µg/g diet) of gossypol in the presence or absence of Cry1Ac after 7 d (Fig. 3a). However, irrespective of strain, larvae feeding on 0.15% gossypol in the presence of 15 µg Cry1Ac toxin/g diet had significantly less gossypol/mg body weight.

Effect of Cry1Ac and Cotton Powder Strain (F_1=32.48, P< 0.001), treatment (F_3=602.14, P<0.001), and the strain X treatment interaction (F_3=71.38, P<0.001) had a significant effect on the percentage of larvae molting to third instar (Table 4). Differences between strains were observed with Cry1Ac toxin (P < 0.001). However, no differences

Table 2 Susceptibility of Cry1Ac-resistant (AR1) and susceptible (SC1) *Helicoverpa zea* to Cry1Ac, and its 1:1 mixture with gossypol

Treatments	Strain	IC_{50} (µg/g diet)[a,b]	Slope	Intercept
Cry1Ac	AR1	17.56 (12.02–23.10)[c]	0.10 (0.05–0.15)	−1.78 (−2.28 to −1.29)
	SC1	7.07 (5.99–8.75)	0.29 (0.22–0.35)	−2.87 (−3.51 to −2.24)
Cry1Ac + Gossypol	AR1	10.04 (8.28–11.27)	0.29 (0.22–0.36)	−2.02 (−2.54 to −1.50)
	SC1	5.41 (4.68–6.14)	0.48 (0.36–0.60)	−2.60 (−3.29 to −1.91)

The test was conducted in three replicates of 32 insects each

[a] IC_{50} values expressed as failure to molt to third instar

[b] IC_{50} values for mixture of Cry1Ac and gossypol are expressed in concentrations of Cry1Ac

Table 3 Weight reduction (EC) of Cry1Ac-resistant (AR1) and susceptible (SC1) *Helicoverpa zea* on Cry1Ac, gossypol, and their 1:1 mixture

Treatments	Strains	EC_{50} (µg/g diet)[b]	EC_{90} (µg/g diet)[b]
Gossypol	AR1	1,171.81 (892.63–1,450.98)[b]	2,109. 25 (1,606.73–2,611.77)
	SC1	1,204.01 (916.35–1,491.66)	2,167.21 (1,649.43–2,684.99)
Cry1Ac	AR1	0.99 (0.12–1.87)	3.30 (0.39–6.20)
	SC1	0.97 (0.31–1.63)	3.23 (1.04–5.42)
Cry1Ac + Gossypol	AR1	1.35 (0.52–2.18)	4.48 (1.72–7.25)
	SC1	0.94 (0.26–1.63)	3.14 (0.86–5.41)

[a] The test was conducted in three replicates of 32 insects each

[b] *EC* Effective concentration (concentration of Cry1Ac) resulting in a 50% (EC_{50}) and 90% (EC_{90}) weight reduction in treated larvae compared to the untreated control group; values for Cry1Ac + gossypol are expressed in concentrations of Cry1Ac

[c] Values in the parenthesis are 95% confidence intervals

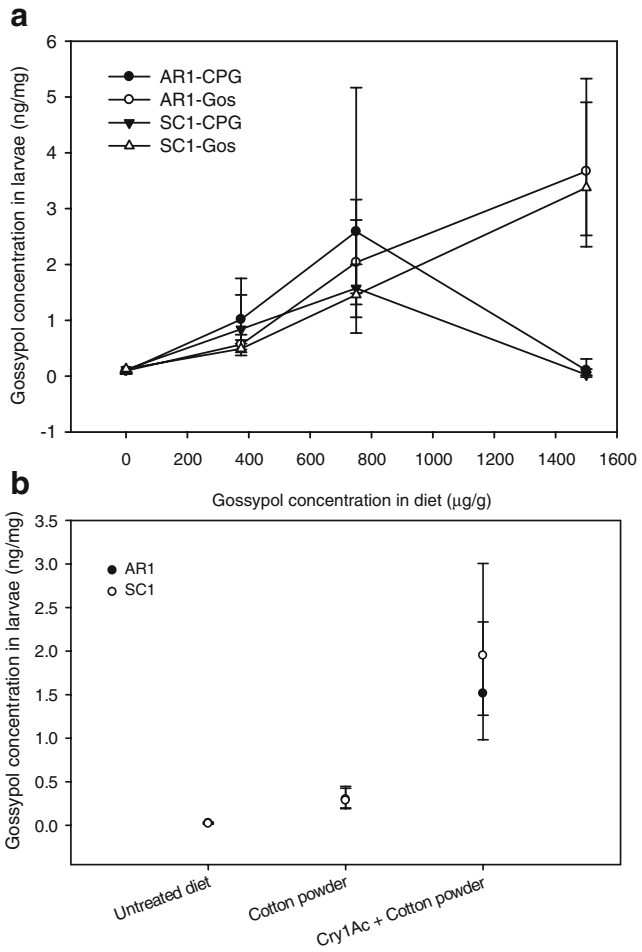

Fig. 3 Concentration of gossypol in Cry1Ac-resistant (AR1) and susceptible (SC1) *Helicoverpa zea*. When fed on **a** different concentrations of gossypol (375 µg/g, 750 µg/g & 1,500 µg/g) in the diet alone and in 1:1combination with Cry1Ac (3.75 µg/g, 7.5 µg/g, 15 µg/g). CPG=Cry1Ac + Gossypol; Gos = Gossypol. **b** 4% cotton powder alone or in combination with 15 µg Cry1Ac/g diet. The data represent the mean of three replications and standard errors are back-transformed values from logarithmic scale

were observed between strains on untreated diet (P=0.933), nor on 4% cotton powder in the presence (P = 0.051) or absence (P=0.262) of Cry1Ac. Gossypol concentration in larvae did not differ between strains (F_1=0.0, P=0.985), and there was no significant interaction with treatment (F_2=0.70, P=0.501). However, larvae feeding on different treatments had significantly (F_2=243.84, P<0.001) different levels of gossypol (Fig. 3b). Treatments (F_3= 103.91, P<0.001), strains (F_1=6.24, P=0.018), and their interactions (F_3=12.35, P<0.001) influenced larval weight significantly (Fig. 4). AR1 and SC1 larval weights differed on Cry1Ac toxin alone (P<0.001), but not in other treatments.

Synergistic Interactions Significant synergistic interactions of Cry1Ac toxin with cotton powder and gossypol were

Table 4 Percent failure to molt to 3rd instar of Cry1Ac-resistant (AR1) and susceptible (SC1) *Helicoverpa zea* on cotton powder in the presence and absence of Cry1Ac toxin

Treatments	N[a]	% Failure to molt to 3rd instar (mean ± SE)		
		AR1	SC1	P value
Untreated diet	192	11.59±2.66[a]	11.98±3.90[a]	0.933
Cry1Ac[b]	200	20.59±3.04[a]	86.98±3.27[b]	< 0.001
Cotton powder[c]	222	22.65±3.99[a]	14.23±5.94[a]	0.262
Cry1Ac + cotton powder	205	98.31±0.69[c]	96.01±1.09[b]	0.051
Strains		F_1=32.48, P<0.001		
Treatment		F_3=602.14, P<0.001		
Strain * Treatment		F_3=71.38, P<0.001		

[a] Number of insects tested; means within a column followed by different superscript letters are significantly different at P=0.05

[b] Cry1Ac used at 15 µg Cry1Ac toxin/g diet

[c] Cotton powder incorporated at 4% wt/wt

observed for AR1-larvae, but not for SC1-larvae when assessing percent failure to molt to third instar (Table 5). However, there was an additive interaction only at lower levels of Cry1Ac and gossypol for both insect strains.

Discussion

When reared on N*Bt* cotton, *Helicoverpa zea* larvae of AR and SC strains did not differ in survivorship nor in time required for pupation. In contrast, Cry1Ac-resistant *H.*

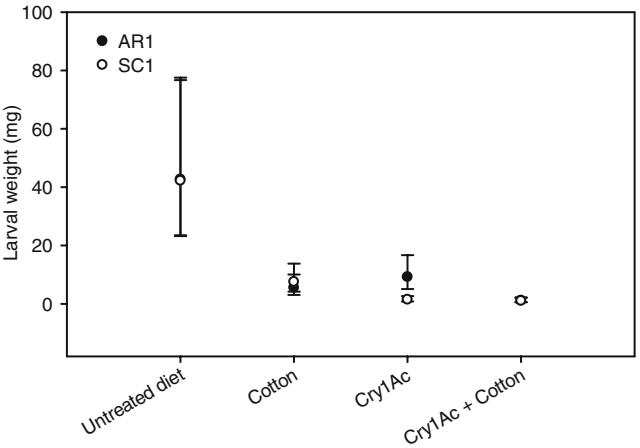

Fig. 4 Effect of 4% cotton powder in the presence and absence of 15 µg Cry1Ac/g diet on larval weight in Cry1Ac-resistant (AR1) and susceptible (SC1) *Helicoverpa zea*. The data represent the mean of three replications and standard errors are back-transformed values from logarithmic scale

Table 5 Interactions of Cry1Ac with gossypol and cotton powder against Cry1Ac-resistant (AR1) and susceptible (SC1) *Helicoverpa zea* as measured by failure to molt into third instars after 7 d

Compounds	Concentration (μg/g)		Strain	N[a]	Failure to molt to 3rd instar (%)		χ^2	Effect
	Cry1Ac	Gossypol			Observed	Expected[b]		
Cry1Ac + Gossypol	15	1500	AR1	96	92.97	37.70	81.02	Synergistic
	7.5	750		96	22.60	15.67	2.13	Additive
	3.75	375		96	6.66	8.32	0.33	Additive
	15	1500	SC1	96	98.92	85.24	2.20	Additive
	7.5	750		96	68.16	61.78	0.66	Additive
	3.75	375		96	36.56	35.17	0.05	Additive
Cry1Ac + Cotton powder (4%)	15	0.006	AR1	222	99.23	22.01	271.04	Synergistic
	15	0.006	SC1	221	95.60	85.15	1.28	Additive

[a] Number of insects tested

[b] Expected mortality is calculated from observed mortalities in different treatments after adjusting for control mortality

virescens (Tabashnik et al. 2003), *H. armigera* (Bird and Akhurst 2004), and *P. gossypiella* (Liu et al. 1999) showed significantly slower larval development on N*Bt* cotton than susceptible individuals (Bird and Akhurst 2004). These results suggest that the *H. zea* AR-strain differs from the *H. zea* SC-strain primarily by the ability to survive higher concentrations of Cry1Ac toxin.

Bt cotton squares used for this study contained commercially acceptable concentrations of Cry1Ac toxin as measured by ELISA. ELISA results are particularly important for this study since cotton Cry1Ac toxin levels may change due to drought or treatment of samples after collection. Indeed, mean Cry1Ac expression levels were reduced by 45% from 86 d to 124 d after planting during a very hot and dry season. Greenplate (1999) found a similar reduction of Cry1Ac expression with the increase in the age of cotton plants. In contrast, refrigeration of harvested squares at 4–7°C for up to 13 d did not result in a significant reduction in Cry1Ac levels. These results may help future investigations in which *Bt* cotton squares will need to be refrigerated for extended periods prior to use.

Cry1Ac toxin was tested at 15 μg/g diet, the minimum concentration observed in lyophilized *Bt* cotton squares by using ELISA. However, fresh *Bt* cotton tissue contains about six to eight fold less Cry1Ac toxin compared to lyophilized tissue (SS unpublished data). Therefore, even at a concentration four to six fold higher than found in fresh *Bt* cotton squares (typically 2–4 μg/g; SS unpublished data), 55% of AR1-larvae could develop to pupation thus suggesting that AR-larvae should be able to develop to pupation on *Bt* cotton especially if larvae feed selectively on tissues that express lower Cry1Ac concentrations (such as bracts) than squares (KJA and WJM unpublished data; Sivasupramaniam et al. 2008). However, no AR-larvae reached pupation on *Bt* cotton even though the AR strain showed significant decreases in mortality compared to SC at 4 d and 7 d. Similar results were found by Tabashnik et al. (2008). Our results suggest that testing the survivability of putative Cry1Ac-resistant insects on tissues that express Cry1Ac for a time that is not sufficient for pupation may overestimate the capability for field-evolved resistance.

Because the nutritional value of plant material is significantly lower than nutritionally-rich artificial diet, larvae can consume up to 6–8-fold more plant material compared to artificial diet (Naeem et al. 1992; Woods 1999). Therefore, both susceptible and resistant *H. zea* would be expected to consume much higher levels of Cry1Ac toxin when feeding on *Bt* cotton squares than with similar toxin concentrations incorporated into diet, thus resulting in much higher than expected mortality (which was observed). This is primarily the reason why a six to eight fold increase in Cry1Ac toxin concentration (15 μg/g diet) was chosen as the highest concentration representing *Bt* cotton squares. When the AR1 strain was reared on 100 μg Cry1Ac toxin/g diet (about 16–42 times the level of Cry1Ac toxin found in *Bt* cotton squares) from 3rd instar to 5th instar after being selected on 500 μg Cry1Ac toxin/gram diet, many larvae died before pupation, and those larvae not dead were severely stunted (WJM, unpublished data). Furthermore, increased consumption of plant secondary compounds such as gossypol (that have been shown to synergize Cry1Ac toxin), in addition to Cry1Ac toxin, could also help explain the increased mortality of AR-larvae on cotton squares as compared to artificial diet. Thus, consideration of the actual amount of insecticidal compounds consumed is critical when comparing toxicities from different diets.

Results from Cry1Ac mixtures with gossypol and cotton powder showed synergistic interactions at the highest concentration tested for AR1, but not for SC1. The highest concentration of gossypol tested (0.15%) was the mean of the two concentrations of gossypol tested by Carrière et al. (2004), and it is representative of the gossypol concentration in cottonseed in Pima and Upland cotton varieties (as discussed in Carrière et al. 2004). In addition, 4% cotton

powder was synergistic in its activity with Cry1Ac toxin against AR1 even though it contained only 6 µg gossypol/g (data not shown). Other than gossypol, cotton plants produce many other insecticidal secondary metabolites such as heliocides H_1 and H_2, and hemigossypolone (Hedin et al. 1991). These compounds also could potentially interact to reduce AR survivorship on *Bt* cotton. Sachs et al. (1996) showed that cotton plants that express high levels of terpenoids (e.g., gossypol) along with Cry1Ab had reduced survivorship of tobacco budworm, *H. virescens*, compared to cotton plants that expressed low to conventional levels of terpenoids. Interestingly, the use of corn powder instead of cotton powder in combination with Cry1Ac also resulted in a synergistic response with Cry1Ac-resistant, but not with susceptible larvae (data not shown), suggesting that a more generalized plant effect (not limited to cotton) is interacting with Cry1Ac against resistant insects. Because *Bt* toxin is known to have antifeedant activity (Whalon and Wingerd 2003), AR-larvae would be expected to consume more diet that contains either Cry1Ac or Cry1Ac + gossypol than SC-larvae, thus agreeing with the observation that synergism between Cry1Ac toxin and gossypol or cotton powder should occur only for AR, However, because no differences in effects of different gossypol concentrations on AR and SC-larvae were observed, the synergism of Cry1Ac toxin and gossypol or cotton powder in AR-larvae should be related to the increased consumption of Cry1Ac toxin, not gossypol.

This is the first report of plant compounds other than protein inhibitors that synergize the activity of *Bt* proteins, especially against Cry1Ac-resistant insects. To date, most compounds that are synergistic with *Bt* Cry proteins are other *Bacillus* spp. or *Bt* products such as spores or spore crystal mixtures (Liu et al. 1998; Moar et al. 1989, 1995a), zwittermicin A (Broderick et al. 2000), β-exotoxin (Moar et al. 1986), CytA (Wirth et al. 1997), and a peptide expressed in *E. coli* that contains a corresponding *Bt* binding sequence (Chen et al. 2007). However, plant protease inhibitors and several chemical insecticides also have been reported to synergize *Bt* proteins (Herfs 1965; MacIntosh et al. 1990a). Gossypol occurs naturally in an enantiomeric mixture of both (+)-gossypol and (−)-gossypol, and the ratio varies among commercial cotton cultivars. Both of these forms reduced the survivorship of *H. zea*, and a racemic mixture of 1:1 had a synergistic effect at 0.16% (Stipanovic et al. 2006). The gossypol obtained from Sigma used in the current study was extracted from cotton seeds, and the ratio of enantiomeric forms of gossypol was not provided. Therefore, further studies are warranted to quantify the ratios of enantiomers and to evaluate their interactions with Cry1Ac.

The synergistic interaction of gossypol and cotton powder with Cry1Ac observed in AR1-larvae may help explain the inability of AR-larvae to survive and produce fertile adults on *Bt* cotton. Carrière et al. (2004) suggested that increased susceptibility of Cry1Ac-resistant *P. gossypiella* to gossypol was linked to the cadherin mutation resistance mechanism (Morin et al. 2003; Carrière et al. 2006). However, AR-larvae have no observable differences in Cry1Ac binding (essentially eliminating a cadherin mutation as a potential resistance mechanism), and appear to have altered proteolysis as a resistance mechanism (Anilkumar et al. 2008a; WJM and KJA unpublished data). Additionally, the AR1 strain was not differentially susceptible to gossypol alone compared to the SC1 strain, nor did AR1-larvae contain more gossypol than SC1-larvae, as has been suggested for Cry1Ac-resistant *P. gossypiella* (Carrière et al. 2004). One possible explanation for synergistic effects of gossypol and Cry1Ac only on AR-larvae is that gossypol is degraded by upregulation of a cytochrome P450 in *Helicoverpa armigera* (Mao et al. 2007). Typically, overexpression of detoxification enzymes results in fitness costs (Matsumura 1985). Assuming that Cry1Ac-resistance in AR-larvae is due to proteolysis, (most likely overexpression of a digestive protease), overexpression of two enzymes may result in increased fitness costs, which were observed in AR-larvae (Anilkumar et al. 2008b). A change of midgut protease activity also could affect nutritional protein digestion which could also influence larval growth, as was observed for AR1 (Gassmann et al. 2009). Because synergism was determined only for AR1-larvae based on their failure to molt to third instar, differences in observed development also could be influenced by changes in hormone [such as juvenile hormone (JH) 1] concentrations caused by competition with gossypol for JH1 binding sites, as has been suggested by Carrière et al. (2004). Therefore, future studies are warranted to determine how resistance mechanisms not associated with binding (or fitness costs involved with Cry1Ac selection) are affected by the presence of gossypol and other plant compounds.

The prediction of field-evolved *Bt* resistance on the basis of laboratory bioassays has always been difficult. Field-evolved resistance has not occurred in *H. zea* even after 12 y of commercial use of *Bt* cotton in the USA (Ali et al. 2006, 2008; Moar and Anilkumar 2007; Moar et al. 2008) even though laboratory experiments have shown that *H. zea* does have the capability to become resistant to the *Bt* protein (Cry1Ac) in *Bt* cotton (Luttrell et al. 1999; Jackson et al. 2004b; Anilkumar et al. 2008a). Results presented in this study help to illustrate that the actual hurdles that *H. zea* must overcome to become resistant to *Bt* cotton in the field are complex, and they help to explain the absence of field-evolved resistance in this pest.

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Oxidative Responses of St. Augustinegrasses to Feeding of Southern Chinch Bug, *Blissus insularis* Barber

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Abstract Southern chinch bug, *Blissus insularis* Barber (Hemiptera: Blissidae), is a serious insect pest of St. Augustinegrass, *Stenotaphrum secundatum* (Walt.) Kuntze, a turfgrass commonly grown in the southeastern United States. Resistance to southern chinch bug has been identified in the polyploid St. Augustinegrass varieties 'Floratam' and 'FX-10', and the diploid 'Captiva'. However, southern chinch bug in Florida and elsewhere has overcome Floratam's resistance. This research investigated the potential role of selected plant oxidative enzymes in resistance/susceptibility to southern chinch bug in two polyploid varieties (FX-10 and Floratam) and two diploid varieties (Captiva and Palmetto). Oxidative enzyme activity was estimated spectrophotometrically from plant samples collected 1, 3, 5, and 8 days after southern chinch bug infestation and from uninfested control plants. Resistant FX-10 and Captiva had significantly higher peroxidase activity, while Captiva had significantly higher polyphenol oxidase activity 5 and 8 days after infestation compared to uninfested controls. FX-10 had higher lipoxygenase activity 3, 5, and 8 days after infestation compared to uninfested controls. Catalase activities did not differ between infested and control plants in any of the varieties tested. Native gels stained for peroxidase indicated that certain isozymes in FX-10 and Captiva were induced 5 and 8 days after infestation. Isozyme profiles of polyphenol oxidase and lipoxygenase did not differ between control and infested FX-10, Floratam, Captiva, and Palmetto. Potential mechanisms to explain the correlation of resistance to southern chinch bug in FX-10 and Captiva with higher activities of oxidative enzymes are discussed.

Keywords Catalase · Host plant resistance · Induction · Lipoxygenase · Oxidative enzymes · Peroxides · Polyphenol oxidase · Southern chinch bug · Hemiptera · Blissidae

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Introduction

St. Augustinegrass, *Stenotaphrum secundatum* (Walt.) Kuntze, is the most widely planted turfgrass in Florida and the southeastern USA. This turfgrass species accounts for 85% of total sod production in Florida, is currently planted on more than one million acres, and is worth more than $262 million annually (Haydu et al. 2005). The southern chinch bug, *Blissus insularis* Barber (Hemiptera: Blissidae), is a major challenge to the production and use of St. Augustinegrass (Crocker 1993), and necessitates numerous insecticide applications annually to maintain the turf's aesthetic value. Unfortunately, this insect has developed resistance to several insecticides (Reinert and Portier 1983; Cherry and Nagata 2005, 2007).

Host plant resistance to southern chinch bug has been an important component of integrated management of this insect for many years. Floratam, a polyploid variety of St. Augustinegrass with resistance to southern chinch bug, was released in Florida and other Gulf Coast states in 1973 (Horn et al. 1973). However, in 1985, a population of

chinch bug in Florida was reported to have overcome Floratam's resistance (Busey and Center 1987); this ability to use Floratam as a host is now widespread throughout Florida (Nagata and Cherry 2003).

As potential alternatives to Floratam, two southern chinch bug resistant St. Augustinegrass varieties were developed: a polyploid line, FX-10 (Busey 1993) and a diploid line, Captiva (Nagata and Cherry 2003). We have shown that both FX-10 and Captiva deter feeding of southern chinch bugs (i.e., through antixenosis) (Rangasamy et al. 2006). Captiva also had adverse effects on the biology of the southern chinch bug (i.e., antibiosis) (Rangasamy et al. 2006), suggesting a metabolic basis for insect resistance.

Currently, the biochemical or physical mechanisms of resistance in these varieties are unknown. Our goal is to decipher mechanisms that will facilitate efforts to breed St. Augustinegrass varieties with durable chinch bug resistance. The mechanisms of plant resistance against phloem-feeding aphids have been studied extensively, compared to other phloem feeders (Thompson and Goggin 2006); however, aphids feed on their host plants by probing intercellularly, while phloem-feeding southern chinch bugs probe intracellularly and, likely, this results in more and different kinds of cellular damage and plant response. Thus, a study of the plant response to chinch bug feeding is warranted. One prominent plant response to insect herbivore attack is the induction of oxidative enzymes, such as polyphenol oxidase (PPO), peroxidase (POX), lipoxygenase (LOX), and catalase (CAT) (Green and Ryan 1972; Hildebrand et al. 1986; Felton et al. 1989, 1994a, b; Stout et al. 1999; Constabel et al. 2000; Chaman et al. 2001; Ni et al. 2001; Heng-Moss et al. 2004). These enzymes, because of their potential roles in plant signaling, synthesis of defense compounds, and/or in oxidative stress tolerance, have been implicated in plant resistance to insect herbivores.

The objective of this investigation was to compare the enzymatic responses of resistant vs. susceptible varieties of diploid and polyploid St. Augustinegrass to feeding by a population of southern chinch bug that has overcome Floratam resistance. Our study focused on the induction of the oxidative enzymes POX, PPO, CAT, and LOX in four cultivars of St. Augustinegrass challenged by adult southern chinch bugs feeding.

Methods and Materials

Plants, Insects, Sample Collection St. Augustinegrass varieties selected for comparison included two diploids (Captiva, chinch bug-resistant, and Palmetto, chinch bug-susceptible) and two polyploids (FX-10, resistant and Floratam, susceptible), all provided by R.T. Nagata (Everglades Research and Education Center, Belle Glade, FL, USA). Rooted sprigs from single node cuttings were grown in plastic pots (12.5 cm diam×12.5 cm depth) containing a 1:1 (*v*/*v*) mixture of sand and Fafard mix #2 (Conrad Fafard, Agawam, MA, USA). Plants were grown in a glass house with ambient light at a temperature of 28°C (24–32°C), 70% RH (65–75% RH) and fertilized with Osmocote Plus 15-9-12 (Scotts-Sierra Horticultural Products Company, Marysville, OH, USA) at the rate of 5 g kg^{-1} of growing medium. Stolons (approx. 30 to 40 cm in length and a minimum of 3 nodes) were used in experiments. Plants were 6–8 week-old.

Southern chinch bugs were collected from Royal Palm Beach (Palm Beach County, FL, USA) for all experiments. Insects were collected by vacuuming southern chinch bug-infested St. Augustinegrass lawns, as described by Cherry (2001). In the laboratory, fifth instars and adults were separated from the debris, placed into 20-l plastic buckets, provided with fresh Floratam runners, and maintained at 18°C in growth chambers until use. Twenty adult chinch bugs (1:1 sex ratio) were confined in organdy sleeve cages on the youngest three nodes of one stolon of a potted St. Augustinegrass plant. Chinch bugs were removed from the stolon before collecting the sample for biochemical analysis. A 2-g amount of a sample of auxiliary shoot (including leaf sheaths and blades) was excised from each stolon and collected 1, 3, 5, and 8 days after chinch bug infestation. Thes amples were frozen immediately in liquid nitrogen and stored at −80°C until analysis. Five replicate plants of each variety were used for each chinch bug infestation period along with an equal number of control plants whose stolons were confined in cages without chinch bugs.

Sample Preparation

Frozen St. Augustinegrass samples were ground into powder in a mortar chilled with liquid nitrogen. Soluble proteins were extracted using HEPES (4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid) buffer (20 mM; 5 ml; pH 7.2) containing protease inhibitors [0.3 ml g^{-1} plant tissue: a mixture of 1 μM bestatin, 0.2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 1 μM pepstatin A, 1 μM E-64, 10 μM leupeptin, 1 mM 1, 10-phenanthroline (Sigma, St. Louis, MO, USA), and 1% (wt/v) polyvinylpyrrolidone]. The homogenate was filtered through a 4X muslin cloth and centrifuged at 10,000×g (10 min at 4°C). The supernatant was collected, desalted using a PD-10 desalting column (Sephadex G-25M, Amersham Biosciences Inc., Piscataway, NJ, USA), and stored at −80°C until protein and enzyme analyses.

Estimation of Total Protein

Total protein content and enzyme activities were determined with a Beckman Model DU 640 Ultraviolet-Visible

spectrophotometer. Total protein content was determined by following the modified method of Lowry et al. (1951) using the Folin-Ciocalteau phenol reagent (Pierce Chemical, Rockford, IL, USA) and bovine serum albumin as a standard.

Enzyme Activities

Soluble POX specific activity was measured with guaiacol (Sigma, St. Louis, MO, USA) as a substrate. Increase in absorbance at 470 nm was monitored for 2 min using a protocol modified from Hildebrand et al. (1986) and Hori et al. (1997). Reactions were started by adding H_2O_2 (30% *v/v*; 10 µl) into a quartz cuvette containing HEPES-KOH (250 mM; 100 µl; pH 7.0), protein extract (5 µl); guaiacol (18 mM; 300 µl), and distilled water (585 µl). POX specific activity (µmoles mg protein^{-1} min^{-1}) was calculated using the molar absorptivity of guaiacol at 470 nm (ε = 26.6M^{-1} cm^{-1}).

Catalase-specific activity was determined following a modified protocol from Hildebrand et al. (1986). The reaction was conducted in a quartz cuvette, started by adding protein extract (200 µl) with HEPES-KOH (250 mM; 100 µl; pH 8.0), H_2O_2 (75 mM; 100 µl), and distilled water (600 µl). The reaction was monitored at 240 nm for 2 min; CAT specific activity was determined using the molar absorptivity of H_2O_2 at 240 nm (ε = 43.6M^{-1} cm^{-1}) and expressed as µmoles mg protein^{-1} min^{-1}.

PPO activity was measured using a protocol modified from Hori et al. (1997). The reaction was started in a cuvette by adding protein extract (25 µl) to HEPES-KOH (250 mM; 100 µl; pH 6.0), catechol (1.6%; 500 µl; Sigma, St. Louis, MO, USA) and distilled water (375 µl). PPO activity was monitored at 470 nm for 2 min and calculated as the change in A_{470} mg protein^{-1} min^{-1}.

LOX activity was determined by using a protocol modified from Skorzynska-Polit and Krupa (2003). Linoleic acid (Fluka, St. Gallen, Switzerland) substrate was prepared by following a protocol described in Koch et al. (1992). A stock solution of sodium linoleate was prepared by mixing linoleic acid (10 mM; 14 µl), Tween-20 (14 µl), and oxygen-free distilled water (2 ml). The mixture produced a milky emulsion after drawing mixing with a Pasteur pipette. The emulsion was cleared by addition of 0.5M NaOH (0.11 ml). The volume was made up to 5 ml with oxygen-free distilled water and dispensed into 2-ml screw-capped vials that were flushed with N_2 and stored at −20°C. The reaction was started in a quartz cuvette by adding linoleic acid (10 mM; 10 µl) to the protein extract (50 µl), HEPES-KOH (250 mM; 100 µl; pH 7.0), and distilled water (860 µl). The change in absorbance at 234 nm was recorded for 5 min. LOX activity was expressed as the change in A_{234} mg protein^{-1} min^{-1}.

Isozyme Profiles

Native gel electrophoresis (Mini-Protean III, Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to study isozyme expression of POX, PPO, and LOX. Equal amounts of protein (30 µg/well) were loaded into discontinuous 10-well polyacrylamide gels [4.5 % (w/v) and 7.5% (w/v)] for POX and PPO, and 5.0% (w/v) and 12.5% (w/v) gels for LOX. Gels were run for 120 min (at 30 mA at 4°C) using a tris-glycine (pH 8.3) continuous buffer for POX and PPO isozymes. The gels containing POX and PPO enzymes were stained using a protocol modified from Vallejos (1983); LOX isozymes were stained according to a protocol by Heinisch et al. (1996).

Gels to be stained for POX activity were incubated in sodium acetate buffer (50 mM, pH 5.0) and run at 25±2°C on a platform shaker (50 rpm). After 10 min of incubation, H_2O_2 (30%; 20 µl) and 4-chloronaphthol (10 mg dissolved in 0.5 ml methanol) were added to the buffer. Peroxidase activity was visualized as dark blue bands after approx. 20 min. Gels stained for PPO activity were soaked in HEPES buffer (20 mM; pH 7.2) containing DL-β-(3,4-dihydroxyphenylalanine DOPA (5 mM; Sigma, St. Louis, MO, USA) for 30 min at 25±2°C on a platform shaker (50 rpm). PPO activity was visualized after 30 min as dark brown bands on a clear background.

Gels to be stained for LOX activity were rinsed briefly in distilled water, incubated (30 min; 4°C) in linoleic acid substrate solution—prepared in the dark by mixing linoleic acid (50 µl) and 95% ethanol (50 µl)—and placed on a platform shaker (50 rpm). The gel was rinsed again in distilled water and incubated on a shaking platform (50 rpm at 25±2°C for 10 min). Gels were stained with sodium borate buffer (0.2 M; 20 ml; pH 9.0) containing *N*, *N*-dimethyl-*p*-phenylenediamine (0.5 g), methanol (4.5 ml), and acetic acid (0.5 ml; 1:1, acid:water), and diluted to 50 ml with distilled water. The isozymes were observed as pink bands.

Statistical Analysis

The study was designed as a completely randomized split-plot in time where insect infestation was the whole-plot treatment, and time after infestation was the sub-plot treatment with five replications. Mean enzyme activities in infested and control plants were compared within ploidy levels (polyploid varieties, FX-10 and Floratam; diploid varieties, Captiva and Palmetto) using PROC GLM (SAS 1999). The means of POX and PPO activities were log (x)-transformed to correct for nonhomogeneity of variances. Tukey's test was used for mean separations.

Results

Total Protein Content

There were no significant differences in total extractable protein between chinch bug-infested and control plants for any of the St. Augustinegrass varieties at any time period after infestation ($P > 0.05$); samples averaged 8.75 mg protein g^{-1} fw.

Peroxidase Activity

Polyploid FX-10 plants infested with southern chinch bug had nearly two-fold higher POX-specific activities 5 and 8 days after infestation compared to uninfested control FX-10 plants and infested and uninfested susceptible Floratam plants ($F_{3,48}$ = 43.20, $P<0.001$) (Fig. 1). There was no interaction between polyploid St. Augustinegrass varieties and infestation period. Similarly, diploid Captiva plants infested with southern chinch bugs had a two-fold increase in POX-specific activities 5 and 8 days after infestation compared to uninfested controls and infested or uninfested southern chinch bug-susceptible Palmetto ($F_{3,48}$ = 27.42, $P<0.001$). There also was no interaction between diploid St. Augustinegrass varieties and infestation period.

Polyphenol Oxidase Activity

There were no differences in PPO specific activity between the infested and control plants of the polyploid varieties, FX-10 and Floratam, at any time period after infestation (Fig. 2). However, PPO specific activity differed among control and infested Captiva and Palmetto ($F_{3,48}$ = 5.32, P= 0.003). The southern chinch bug-infested Captiva plants had higher PPO activity 5 and 8 days after infestation compared to their respective control plants. However, there were no differences in PPO activities among infested and control Palmetto plants.

Lipoxygenase Activity

Polyploid FX-10 plants infested with southern chinch bug had higher LOX specific activity 3, 5, and 8 days after infestation compared to their respective control plants and Floratam plants, whether infested or not ($F_{3,48}$ = 485.26, $P<0.001$) (Fig. 3). There was an interaction between polyploid St. Augustinegrass variety and duration of infestation ($F_{9,48}$ = 36.31, $P<0.001$); LOX specific activity in southern chinch bug-infested FX-10 plants peaked 3 days after infestation. It was 2-fold higher than 1 day after infestation, and then declined 5 and 8 days after infestation. The LOX specific activities in southern chinch bug-infested and control Floratam plants were not significantly different. However, chinch bug-infested FX-10 plants had higher LOX activity than uninfested FX-10 controls, Floratam controls, and infested plants 1 and 3 days after infestation. There were no differences in LOX specific activities between infested and control plants of the diploid varieties, Captiva and Palmetto, at any of the time periods studied.

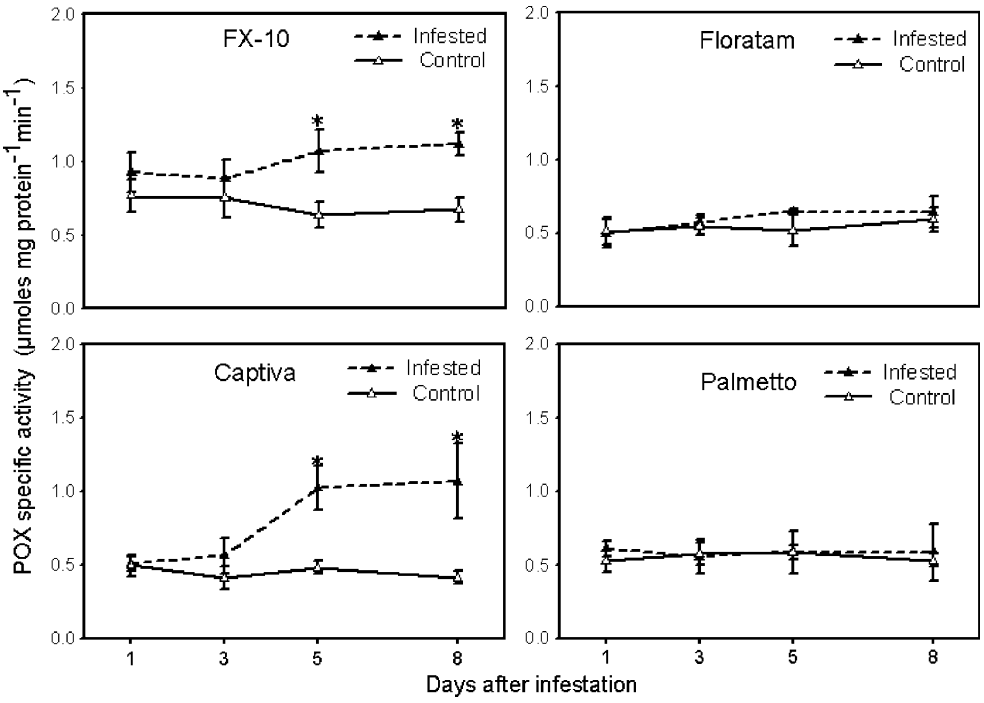

Fig. 1 Levels of peroxidase (POX) specific activity in polyploid (FX-10 and Floratam) and diploid (Captiva and Palmetto) St. Augustinegrass varieties 1, 3, 5, and 8 days after initiation of southern chinch bug feeding. Values are means ± SEM of five replicates. Means marked with * differ significantly between control and infested treatments ($P<0.05$, Tukey's test)

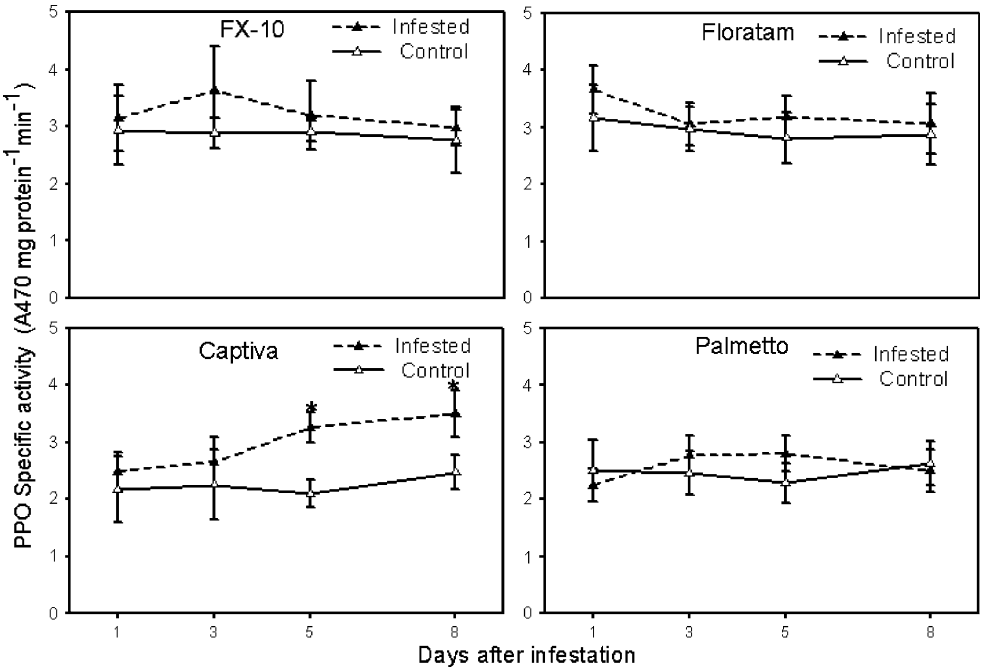

Fig. 2 Levels of polyphenoloxidase (PPO) specific activity in polyploid (FX-10 and Floratam) and diploid (Captiva and Palmetto) St. Augustinegrass varieties 1, 3, 5, and 8 days after initiation of southern chinch bug feeding. The values shown are means ± SEM of five replicates. Means marked with * differ significantly between control and infested treatments ($P<0.05$, Tukey's test)

Catalase Activity

There were no differences observed in CAT specific activity between southern chinch bug-infested and control plants at any time period after infestation in any of the St. Augustinegrass varieties studied ($P>0.05$, data not shown). The mean CAT specific activity in infested and control plants of the four St. Augustinegrass varieties was 5.99 μmoles mg protein^{-1} min^{-1}.

Isozyme Profile Studies

Native gels stained for POX-specific activity showed 5 and 3 isozymes in FX-10 and Captiva, respectively; two of them were induced 5 and 8 days after infestation compared to their respective controls (Fig. 4). There were 3 POX isozymes in Palmetto and 5 in Floratam with no visible difference between infested and control plants at any of the times after infestation (Fig. 4).

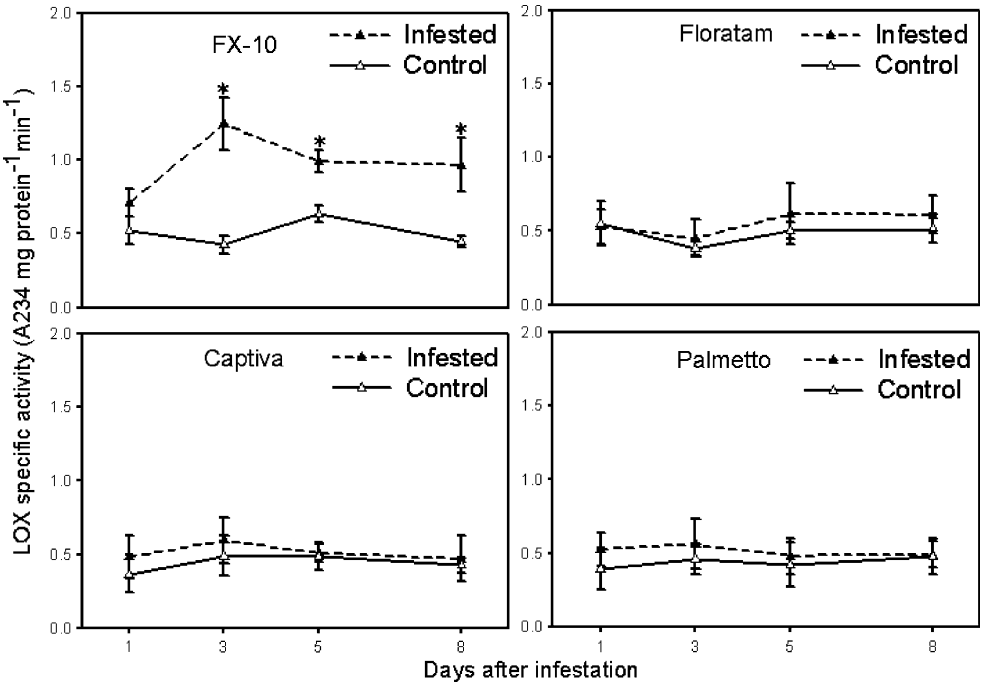

Fig. 3 Levels of lipoxygenase (LOX) specific activity in polyploid (FX-10 and Floratam) and diploid (Captiva and Palmetto) St. Augustinegrass varieties 1, 3, 5, and 8 days after initiation of southern chinch bug feeding. Values are means ± SEM of five replicates. Means marked with * differ significantly between control and infested treatments ($P<0.05$, Tukey's test)

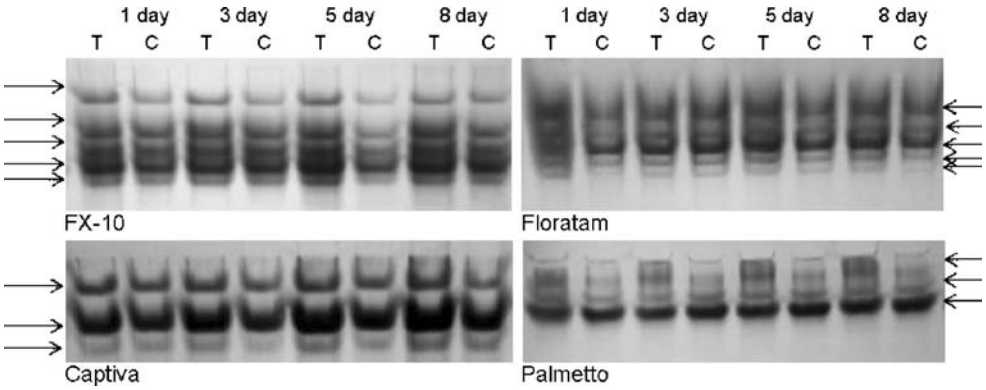

Fig. 4 Native gels stained for POX activity in polyploid (FX-10 and Floratam) and diploid (Captiva and Palmetto) St. Augustinegrass varieties 1, 3, 5, and 8 days after initiation of southern chinch bug feeding. T and C refer to the infested (i.e., treated) and control samples, respectively. Arrows indicate POX isoforms

Native gels stained for PPO activity showed 2 isozymes in Captiva, Palmetto, and Floratam and 3 in FX-10 with no apparent difference between southern chinch bug-infested and control plants at any of the time periods studied (Fig. 5). There were 3 LOX isozymes observed in FX-10, 2 in Captiva and Palmetto, and one in Floratam (Fig. 6). There were no apparent differences in LOX isozyme intensity or number between southern chinch bug-infested and controls plants.

Discussion

Infestation by southern chinch bugs neither increased nor decreased the total extractable protein content of four St. Augustinegrass varieties when measured 1, 3, 5, or 8 days after infestation. Our results agree with those of Heng-Moss et al. (2004) who found no increase in total protein content of resistant buffalograss varieties (Cody and Tatanka) after infestation by the western chinch bug, *Blissus occiduus* Barber. However, Ni et al. (2001) reported an increase in total protein in wheat varieties infested with the Russian wheat aphid, *Diuraphis noxia* (Mordvilko), and the corn leaf aphid, *Rhopalosiphum padi* (L.), in contrast to an earlier report of a two-fold decrease in total protein 2 days after infestation by *D. noxia* (van der Westhuizen and Pretorius 1995). The difference in results may be due to different methods used for determining protein concentration.

We found that both chinch bug-resistant varieties, FX-10 and Captiva, had a nearly two-fold increase in POX specific activity 5 and 8 days after infestation compared to uninfected control plants. Our results agree with many previous reports that POX specific activity increases after herbivore infestation (Hildebrand et al. 1986; Felton et al. 1994a, b; van der Westhuizen et al. 1998; Chaman et al. 2001; Ni et al. 2001; Heng-Moss et al. 2004; Khattab 2007). In contrast, while lupin varieties resistant to green peach aphid, *Myzus persicae* (Sulzer), had significantly different constitutive levels of POX and PPO activity, protease inhibitors and soluble phenolics, the levels of PPO and POX were not increased in response to aphid infestation (Cardoza et al. 2005).

Herbivore damage induces production of H_2O_2 in plants (Dowd and Lagrimini 1997). Oxidative enzymes such as POX and CAT regulate the level of H_2O_2, and POX activity is dependent on the supply of H_2O_2 *in vitro* (Duffy and Felton 1991). A high level of H_2O_2 produced in response to mechanical wounding of tomato plants acted as a secondary messenger and activated later-expressed defense genes,

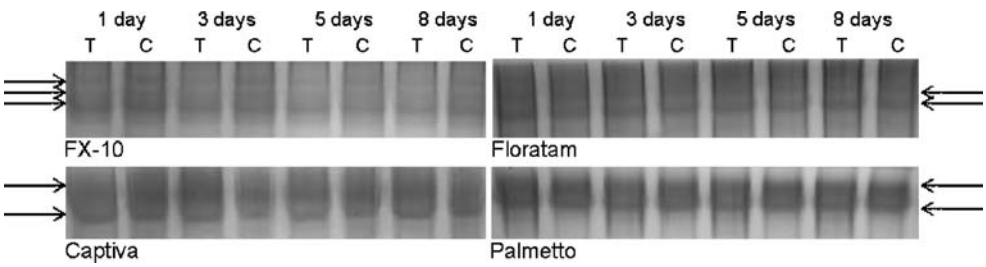

Fig. 5 Native gels stained for PPO activity in polyploid (FX-10 and Floratam) and diploid (Captiva and Palmetto) St. Augustinegrass varieties 1, 3, 5, and 8 days after initiation of southern chinch bug feeding. T and C refer to the infested (i.e., treated) and control samples, respectively. Arrows indicate PPO isoforms

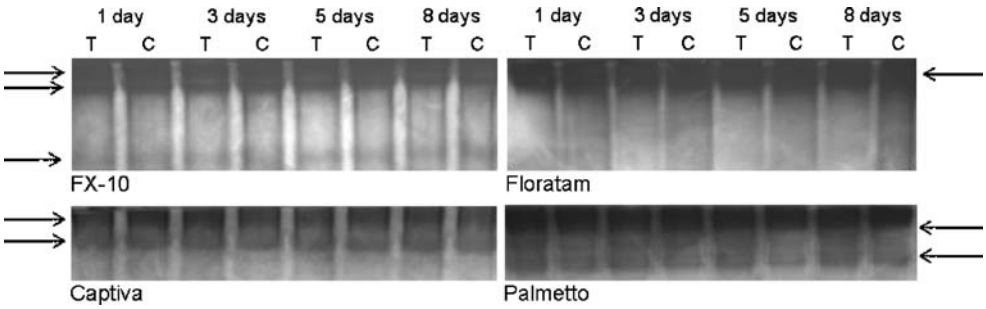

Fig. 6 Native gels stained for LOX activity in polyploid (FX-10 and Floratam) and diploid (Captiva and Palmetto) St. Augustinegrass varieties 1, 3, 5, and 8 days after initiation of southern chinch bug feeding. T and C refer to the infested (i.e., treated) and control samples, respectively. Arrows indicate LOX isoforms

such as those coding for proteinase inhibitors and polyphenol oxidase (Orozco-Cardenas et al. 2001). An increase in POX activity in insect-resistant plants may help to detoxify the peroxides, thus reducing plant tissue damage when compared to susceptible plants (Hildebrand et al. 1986). Peroxidase enzymes are involved with plant cell wall-building processes, by mediating the oxidation of hydroxycinnamyl alcohols into free radical intermediates, cross-linking of polysaccharides and extensin monomers, lignification, and suberization (Chittoor et al. 1999). Brisson et al. (1994) suggested that oxidative cross-linking of the plant cell wall could enhance its ability to slow pathogen ingress. Such a reinforcement of the cell wall in response to insect feeding may serve as a mechanical barrier and prevent insect stylets from reaching their feeding sites (i.e., phloem sieve elements in the case of southern chinch bug). Enhancement of POX isozyme activity in maize (*Zea mays* L.) increased resistance to herbivores and pathogens (Dowd and Lagrimini 1997).

Plant catalases scavenge mitochondrial and peroxisome H_2O_2, and reduce it to H_2O and O_2. Zhu-Salzman et al. (2004) found that CAT genes were down-regulated in resistant sorghum infested with greenbug, and speculated that reduced CAT activity could help the plant maintain high H_2O_2 levels that damage the insect midgut. Park et al. (2006) also found that CAT genes were down-regulated in greenbug resistant sorghum varieties. However, Heng-Moss et al. (2004) reported a loss in CAT activity in response to *B. occiduus* on susceptible buffalograss varieties. Our study found no association between CAT-specific activity levels and southern chinch bug resistance in St. Augustinegrasses, and supports previous reports that used other plant-insect combinations that also indicated no relationship between CAT activity and resistance (Faccioli 1979; Matkovics et al. 1981; Hildebrand et al. 1986).

The association of PPO activity with host plant resistance to insects occurs in many plants including tomato, potato, coffee, and poplar (Duffy and Felton 1991; Constabel et al. 1996; Chaman et al. 2001; Wang and Constabel 2004; Thipyapong et al. 2006). In our studies, only Captiva had higher levels of PPO specific activity as a result of southern chinch bug infestation, whereas FX-10 showed no induction. Heng-Moss et al. (2004) found no association between PPO activity in buffalograsses and their resistance to *B. occiduus*.

Our studies indicate that FX-10 had a two-fold higher LOX-specific activity 3 days after southern chinch bug infestation, and a reduced, but still higher activity 5 and 8 days following infestation compared to control plants (Fig. 4). Increased LOX activity has been reported in soybean in response to two-spotted spider mite infestation (Hildebrand et al. 1986) and attack by *Helicoverpa zea* larvae (Boddie) (Felton et al. 1994a). Increased levels of LOX activity caused oxidative damage in midgut epithelial cells of *H. zea* larvae and reduced their growth (Felton et al. 1994a). Bi et al. (1997) showed an increase in LOX in *H. zea* damaged cotton squares along with altered phenolic contents. Gene expression studies showed that LOX transcripts were elicited in tomato plants in response to attack by the aphid *Macrosiphum euphorbiae* (Thomas) and *M. persicae* (Fidantsef et al. 1999). Gene expression was locally upregulated in *Nicotiana attenuata* Torrey ex Watson after *Macrosiphum nicotianae* Blackman infestation (Voelckel et al. 2004). LOX-deficient *N. attenuata* plants, when planted into native habitats, became more vulnerable to adapted herbivores such as *Manduca sexta* (L.) and attracted new opportunistic herbivores, such as *Empoasca* spp. leafhoppers (Kessler et al. 2004).

Lipoxygenases are ubiquitous enzymes that catalyze the hydroperoxidation of polyunsaturated fatty acids having *cis, cis*-pentadiene moieties (Hildebrand et al. 1986). Linoleic acid and linolenic acid are the major LOX substrates in plants (Hildebrand et al. 1986). Lipid peroxidation by LOX results in formation of fatty acid hydroperoxides that may be degraded to highly reactive aldehydes, γ-ketols, and epoxides (Gardner 1991), hydroxyl radicals, singlet oxygen, superoxide ion and peroxyl, acyl, and carbon-centered radicals (Kanofsky and Axelrod 1986) either through enzymatic or chemical means.

Gardner (1979) mentioned that the interactions between lipid hydroperoxides and proteins results in protein-protein cross linking, and amino acid damage. These chemical changes in proteins may affect the assimilation of insect amino acids. Lipid peroxidation end products could be deterrent to insect feeding and operate via antixenosis, or be toxic and function via antibiosis (Felton et al. 1994a). Both modalities of resistance occur in St. Augustinegrass against the southern chinch bug (Rangasamy et al. 2006). Shukle and Murdock (1983) demonstrated that LOX could act directly as a defense against insect attack in soybean. Lipoxygenases also are involved in inducible herbivore resistance. Oxidation of linolenic acid by LOX is the first step in the jasmonic acid signaling pathway, which has been implicated in the coordination of direct plant defense [e.g., production of oxidative enzymes and protease inhibitors (Staswick and Lehman 1999; Mao et al. 2007)] and indirect plant defense [e.g., production of volatiles (Dicke et al. 1999; Thaler 1999)]. Rayapuram and Baldwin (2006) investigated the performance of *M. sexta* larvae on *N. attenuata* plants silenced in LOX3 expression and jasmonic acid signaling. They found that LOX3-mediated defense in wild plants reduced larval growth, consumption, and frass production compared to the mutant. Mao et al. (2007) transformed maize plants with the wheat oxalate oxidase gene and reported that the up-regulated LOX transcripts and 14-fold higher free phenolics in transgenic plants were positively associated with resistance to the European corn borer (*Ostrinia nubilalis* H.) compared to control plants.

While enzyme induction has been examined in this work, other biochemical adaptations including enzyme inhibition, activation, and substrate availability also may be important factors in determining this trait. We associate the increased levels of POX and LOX in FX-10 and POX and PPO in Captiva with southern chinch bug resistance in these varieties. Increased POX activity seems to be due to the induction of specific isozymes. The fact that enzyme induction differs between the two resistant cultivars suggests that the mechanisms of resistance also differ. This corroborates our finding (Rangasamy et al. 2006) that while FX-10 shows antixenosis to southern chinch bug, Captiva exhibits both antixenosis and antibiosis. Together, our results support the hypothesis that oxidative enzymes play a vital role in herbivore resistance in plants. Our data suggest that identification of genes that encode inducible PPO, POX, and LOX enzymes could be the first step in understanding southern chinch bug resistance in St. Augustinegrass and will be valuable markers for breeding southern chinch bug resistant varieties. Our research supports the hypothesis that chinch bug resistance in St. Augustinegrass has a metabolic basis and highlights the possible involvement of lignification and oxidative enzymes in secondary product synthesis for chinch bug resistance in St. Augustinegrass.

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Interactive Influence of Leaf Age, Light Intensity, and Girdling on Green Ash Foliar Chemistry and Emerald Ash Borer Development

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Abstract Biotic and abiotic environmental factors affect plant nutritional quality and defensive compounds that confer plant resistance to herbivory. Influence of leaf age, light availability, and girdling on foliar nutrition and defense of green ash (*Fraxinus pennsylvanica* Marsh) was examined in this study. Longevity of the emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), adults reared on green ash foliage subjected to these factors was assayed. Mature leaves generally were more nutritious with greater amino acids and a greater ratio of protein to non-structural carbohydrate (P:C) than young leaves, in particular when trees were grown in shade. On the other hand, mature leaves had lower amounts of trypsin and chymotrypsin inhibitors, and total phenolics compared to young leaves. Lower defense of mature leaves alone, or along with higher nutritional quality may lead to increased survival and longevity of emerald ash borer feeding on mature leaves. Sunlight reduced amino acids and P:C ratio, irrespective of leaf age and girdling, and elevated total protein of young foliage, but not protein of mature leaves. Sunlight also dramatically increased all investigated defensive compounds of young, but not mature leaves. Girdling reduced green ash foliar nutrition, especially, of young leaves grown in shade and of mature leaves grown in sun. However emerald ash borer performance did not differ when fed leaves from trees grown in sun or shade, or from girdled or control trees. One explanation is that emerald ash borer reared on lower nutritional quality food may compensate for nutrient deficiency by increasing its consumption rate. The strong interactions among leaf age, light intensity, and girdling on nutrition and defense highlight the need for caution when interpreting data without considering possible interactions.

Keywords *Fraxinus pennsylvanica* · *Agrilus planipennis* · Abiotic factors · Foliar nutrition · Plant defense

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Introduction

Low nutritional quality and occurrence of plant secondary metabolites in many cases contribute to plant resistance to herbivory. For example, herbivores that feed upon plants with higher nutritional quality generally grow faster and perform better (i.e., have greater reproductive potential and longevity) than those reared on lower quality plants (Mattson 1980; Chen et al. 2008a. In addition, many plant allelochemicals adversely affect herbivore colonization and life histories (Ryan 1990).

Levels of nutrients and defensive compounds change in response to biotic and abiotic factors. One factor is age of leaves. Young and expanding tissues generally are believed to contain higher concentrations of nutrients because these tissues require more resources to support their rapid growth (Mattson 1980; Harper 1989). Regarding defenses, it is widely accepted that plant parts with higher fitness values should be better defended (Optimal Defense theory; McKey 1979). The greater fitness value of young expanding leaves over older leaves has been demonstrated experimentally in some plants (McKey 1979; Strauss et al. 2004), as has the elevated accumulation of chemical defenses in these leaves (Ohnmeiss and Baldwin 2000; Chen et al. 2008b).

Light is another factor that may affect plant nutritional and defensive compounds. Reduced light intensity results in low absorbance of solar energy in the light-dependent stage of plant photosynthesis. The resultant low storage of chemical energy may impair capture of CO_2 from the atmosphere and its conversion to photosynthates in the light-independent stage, and subsequent allocation of photosynthates to growth and defense. Plant response to a shaded environment is similar to 'carbon stress' described by the carbon-nutrient balance (CNB) hypothesis (Bryant et al. 1983) an idea that has increasingly lost credibility in recent years (Hamilton et al. 2001; Cipollini et al. 2002; Koricheva 2002; Nitao et al. 2002). According to this hypothesis, trees grown under shaded environments would be more carbon-limited and have lower amounts of carbon-based defensive compounds, while amounts of nitrogen-based defensive compounds would be higher.

Girdling, removal of a ring of bark and phloem around the outer circumference, is a practice used worldwide in agriculture, horticulture, and forestry, with various purposes (Noel 1970; Lahav et al. 1986; Mostafa and Saleh 2006). Girdling interrupts the transport of photosynthates to the roots and has a variety of physiological and biochemical effects on plants such as accumulation of carbohydrates above the girdle and a decline of carbohydrates below the girdle (Noel 1970; Roper and Williams 1989; Li et al. 2003; Mostafa and Saleh 2006). An accumulation of amino acids above the girdle has been reported in *Salix fragilis* twigs (Mittler 1958). Plants are also known to induce defensive chemicals following mechanical wounding and insect herbivory (Stout et al. 1998; Reymond et al. 2000; Lawrence and Koundal 2002), although the effects of girdling on the induction of defensive compounds are little known. Therefore, girdling might alter plant resistance to herbivory due to changes in nutritional qualities and defense.

In this study, we examined the effects of leaf age, light intensity, and girdling on green ash (*Fraxinus pennsylvanica* Marsh) resistance to emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), from a nutritional and defensive chemistry perspective. EAB is an exotic species first detected around Detroit Michigan, USA and Windsor Ontario, Canada in 2002, and is threatening the ash resource in North American (Haack et al. 2002; Poland and McCullough 2006). EAB attacks ash trees (*Fraxinus* spp.) of all species and sizes from large mature trees to trees that are 2 cm in diameter (Cappaert et al. 2005). Adults feed on foliage, and females lay eggs in bark cracks and crevices. The larvae tunnel in the cambial region disrupting the flow of nutrients and girdling the tree. Progress has been made on EAB host range (Anulewicz et al. 2007, 2008), biology (Bauer et al. 2004; Cappaert et al. 2005), behaviors (Rodriguez-Saona et al. 2006; Lelito et al. 2007), and chemical ecology (Rodriguez-Saona et al. 2006; Eyles et al. 2007; Crook et al. 2008; de Groot et al. 2008). EAB adults have been observed to prefer trees grown in open areas over those in the shady ones (Poland et al. 2005). Girdled trees are more attractive to EAB than non-girdled trees (Poland et al. 2005; McCullough et al. 2006, 2009). However, little is known to date about mechanisms that underlie these EAB behaviors and ash tree resistance to EAB, except that certain phloem phenolics might account for the relatively higher resistance of Manchurian ash (*F. mandshurica*) to EAB (Eyles et al. 2007). Elucidation of these mechanisms contributes to ash tree breeding programs to develop resistant varieties or hybrids.

We quantified the nutritional and defense compounds trypsin and chymotrypsin inhibitors, and total phenolics of green ash foliage, and we evaluated adult EAB performance reared on green ash foliage subject to various biotic and abiotic factors. Specifically we tested the following hypotheses: (1) young leaves have higher nutritional qualities and greater defensive compounds than mature leaves; (2) shaded trees have higher foliar nutrients and nitrogen-based defensive compounds (i.e., proteinase inhibitors) but lower carbon-based defensive compounds (phenolics), according to the CNB hypothesis; (3) girdled trees have elevated carbohydrate levels and defensive protease inhibitors and phenolics, but reduced P:C ratio; and 4) adult EAB will have greater longevity on leaves with higher nutritional quality and lower defense compounds.

Materials and Methods

Green Ash Green ash seedlings (2-yr-old and ca. 15–30 cm tall) were used for the ease of manipulation of the treatments. Seedlings were purchased from Lawyer Nursery Inc. (West Plains, MT, USA) in February, 2008, and stored in a dark room at 4°C until potting. Seedlings were potted with TPOT2 tree pots (Width: 15 cm; Height: 41 cm; Volume: 6.23 L; Stuewe & Sons, Inc., Corvallis, OR, USA) using BACCTO® High Porosity Professional Planting Mix (Michigan Peat Co., Houston, TX, USA) and soil at a ratio of 3:1 as planting medium. Seedlings were grown during July–August, 2008 (ca. L16:D8 h) in a greenhouse set at approximately 25±2°C and supplemented with 400-Watt high-pressure sodium lamps from 10:00 pm to 6:00 am. The greenhouse is located on campus of Michigan State University, East Lansing, MI, USA. Osmocote 14-14-14 Classic fertilizer (Scotts Company LLC., Marysville, OH, USA) was mixed with the planting medium at ca. 4 kg m^{-3} soil. Seedlings were watered as need.

Approximately 5 weeks after potting when seedlings had 4–6 fully expanded compound leaves and 3–6 expanding leaves, seedlings were subjected to light intensity (2 levels:

Shade and Sunny) and girdling (2 levels: Girdled and un-girdled) treatments. Shade treatment was a 1.2×0.7×1.1 m (L×W×H) cage built with 1.9 cm (inner diam) PVC pipes (Charlotte, NC, USA) covered with 80% TapeKnit shade cloth (American Nettings & Fabric Inc., Ferndale, WA, USA). The shade cloth reduced the amount of sunlight available to the trees by approximately 70–80%. The sunny treatment did not have a shade cloth cage. Two light intensity levels were assigned randomly to four whole plots arranged in a straight line. Twenty seedlings of similar size were randomly assigned to each of the four whole plots. Half (10) of the seedlings assigned to each whole-plot were randomly selected and girdled (i.e., a 2.5 cm band of phloem removed on the stem at a height of 15 cm from the soil), and the other half were left un-girdled. At the end of the experiments (2 weeks following these treatments), one young (Young: expanding leaves less than 3-weeks-old) or one mature (Mature: fully expanded leaves that were 4- to 6-weeks-old) compound leaf was randomly collected from each plant. Therefore, the experimental design was a completely randomized split-plot design with light intensity as whole plots. The split plots were girdling and leaf age arranged as a 2×2 factorial.

Insects EAB infested ash trees were felled at the end of 2007 and early 2008 and cut into 90-cm long logs. Logs were held in cold storage at 4°C. As beetles were required for experiments, they were allowed to emerge in rearing tubes at 25°C. Upon emergence they were separated by sex and used for bioassay.

Effects on F. pennsylvanica Growth and Leaf Chlorophyll Contents To examine the effects of shade and girdling on green ash growth, heights (from soil to meristem) of green ash seedlings were determined at the onset of treatments and again at the end (2 weeks following treatments). Because leaf chlorophylls are the most important photosynthetic pigments of higher plants and are positively correlated to photosynthetic potential (Filella et al. 1995; Markwell et al. 1995; Richardson et al. 2002), leaf chlorophyll content was measured by using a Minolta SPAD-502 (Konica Minolta Sensing, Inc., Japan) at the end of experiments. Chlorophyll content (μmol m^{-2}) was calculated according to a standard curve for corn (*Zea mays*) plants, chlorophyll (μmol m^{-2})=10 $^{(M^{\wedge}\ 0.261)}$, where M is the chlorophyll meter reading (Markwell et al. 1995). The experiment was a split-plot design as described above.

Effects on F. pennsylvanica Nutritional Chemistry Seedlings were grown as described above. Approximately 2 weeks after treatments, one mature (3–4 weeks after full expansion) or one young (within 2 weeks following sprouting; ca. 20–50% size of mature leaflets) compound leaf from each tree was cut and flash frozen in liquid nitrogen. Samples were brought to the laboratory in a cooler containing dry ice, and 2–4 leaflets of the collected compound leaf were ground to fine powder with mortar and pestle in liquid nitrogen. The ground plant tissue was stored at −20°C until bioassayed.

Approximately 40–80 mg of ground plant tissue were used for extraction. Extraction of total soluble protein, amino acids, and soluble non-structural carbohydrate (glucose, fructose, and sucrose) followed Bi et al. (2003) and Chen et al. (2009). Extraction of non-soluble non-structural carbohydrate (starch) followed Marquis et al. (1997) and Chen et al (2009). Total protein content was determined following the Bradford protein assay, and calculated with a standard curve established by using bovine serum albumin (Ni et al. 2001). Amino acid content was colorimetrically determined using the cadmium (Cd)-ninhydrin procedure (Doi et al. 1981). The amino acid content was calculated from a standard curve generated using glycine as a standard. Glucose, fructose, and sucrose contents were determined by using glucose assay kit (Sigma-Aldrich, St. Louis, MO, USA) and calculated from a standard curve generated using D-glucose as a standard. The starch content was estimated in glucose equivalents. All chemicals used in this experiment and the following were purchased from Sigma-Aldrich (St. Louis, MO, USA), or Fisher Scientific (Pittsburgh, PA, USA).

Effects on F. pennsylvanica Protease Inhibitors and Total Phenols Leaf samples were collected and prepared as in the preceding experiment. Trypsin and chymotrypsin inhibitors extraction followed Stout et al. (1998). Ground and weighed plant tissue was mixed with a Tris-HCl buffer (pH 7.8) containing 7% polyvinylpolypyrrolidone, 1.67 mM phenylthiourea, 0.3 mM KCl, and 0.4 mM ascorbic acid. The mixture was centrifuged at 10,000×g for 10 min. The supernatant was used for assay.

The trypsin inhibitor assay followed Walsh and Wilcox (1970), and Broadway (1993). Bovine trypsin (0.2 mg ml^{-1} in 1 mM HCl) was mixed with an equal volume of plant extract and incubated at room temperature for 10 min. Eighty microliters of the mixture was added to 3 ml substrate solution [1.04 mM *Nα-p*-tosyl-L-arginine methyl ester (TAME) in 0.05 M Tris-HCl buffer]. The activity of trypsin that remained after reaction with trypsin inhibitors in the sample was monitored at 247 nm for 3 min with a Shimadzu UV Mini-1240 spectrophotometer at 25°C and was converted to amount of trypsin from a standard curve using Bovine trypsin within the linear range (0~0.1 mg ml^{-1}). The amount of trypsin inhibitors was expressed as amount of inhibited trypsins, and was calculated by subtracting the trypsins remaining after the

reaction from the amount of trypsin (8 μg) used in the beginning of assay.

The chymotrypsin inhibitor assay followed Walsh and Wilcox (1970), and Broadway (1993). *Nα*-Tosyl-Lys-chloromethylketone (TLCK)-treated bovine chymotrypsin (0.2 mg ml^{-1} in 1 mM HCl) was mixed with an equal volume of plant extract and incubated at room temperature for 10 min. Eighty microliters of the mixture were added to 3 ml substrate solution containing 0.5 mM *N*-benzoyl-L-tyrosine ethyl ester (BTEE), 5% methanol, and 0.05 M Tris-HCl buffer. The activity of chymotrypsin that remained after the reaction with chymotrypsin inhibitors was monitored at 256 nm for 3 min with a Shimadzu UV Mini-1240 spectrophotometer at 25°C and was converted to amount of chymotrypsins from a standard curve using Bovine chymotrypsin within the linear range (0~0.1 mg ml^{-1}). The amount of chymotrypsin inhibitors was expressed as amount of inhibited chymotrypsins, and was calculated by subtracting chymotrypsins remaining after the reaction from the amount of chymotrypsin (8 μg) used in the beginning of assay.

Total phenol extraction followed Hagerman (1988) and Cork and Krockenberger (1991). Determination of total phenol followed Graham (1992). Briefly, ground and weighed plant tissue was extracted ×3, each time with 1 ml 70% acetone at 4°C in the dark for 30 min. The supernatant after each centrifugation was pooled and its concentration [m*mol* g^{-1} fresh weight (FW)] determined using a modified Prussian blue assay. An external standard curve with gallic acid within the linear range was constructed to calculate sample concentration.

EAB Performance in Response to Green Ash Foliage To evaluate effects of leaf age, shade, and girdling on green ash foliar nutritional and defensive chemistry, adult EAB longevity reared on foliage subject to these treatments was assayed. One newly emerged adult EAB male and one female were placed in a sealed 295-ml plastic drinking cup where they were fed excised compound leaves from different treatments (treatments were as described above for the previous experiment). A 5×7 cm area on the side of the cup was cut away and sealed with 1×1 mesh for ventilation. The petioles of excised leaves were placed into 20-ml glass vials filled with water before being placed into cups. Leaves were exchanged with fresh leaves of the same treatment every 2–3 days. Survival of EAB was monitored daily until all EAB were dead. Each treatment was replicated ten times (cups). Cups were kept in chambers set at 25±2°C and 70% relative humidity with a photoperiod of L14:D10h.

Statistical Analyses All experiments were completely randomized split-plot designs with light intensity as whole plots, and girdling and leaf age (arranged as a 2×2 factorial) as split plots. Leaf cholorphyll, foliar total proteins, amino acids, TNC, protein:TNC ratio, trypsin inhibitors, chymotrypsin inhibitors, total phenolics, and EAB longevity were analyzed by PROC MIXED in SAS (SAS Institute 1999) with the interaction of light intensity and its replication as a random effect. Protein:TNC ratio data were log transformed before being subject to analysis. Survival of EAB adults was analyzed with repeated measure ANOVA (PROC GLM in SAS) with date as the repeated measure. Comparison was separately conducted between Young and Mature leaf treatments, between Sunny and Shady treatments, and between Control and Girdled treatments. In all analyses, the type I error α was controlled at 0.05.

Results

Effects on Green Ash Growth and Leaf Chlorophyll Content Green ash seedling height was not affected by light intensity or girdling (data not shown). Effects of leaf age, light condition, and wound status on leaf chlorophylls are shown in Table 1. Mature leaves had higher leaf chlorophyll than young leaves. For mature leaves, exposure to sunny conditions decreased leaf chlorophyll levels, while girdling had no effect. For young leaves, sunny conditions decreased leaf chlorophyll levels of girdled trees, but had no effect on chlorophylls of control trees; girdling decreased chlorophylls of trees under sunny conditions, while it did not affect chlorophylls of trees under shady conditions.

Effects on Green Ash Foliage Nutritional Chemistry Total proteins, amino acids, TNC, and P:C in relation to leaf age, light, and girdling treatments are shown in Table 2. Leaf age and girdling did not affect the amount of proteins. Exposure to sunny conditions increased proteins of young but not mature leaves. Mature leaves had significantly greater amino acids than young leaves, regardless of light and girdling treatments. Shading increased amino acid quantity, while girdling did not affect amino acids.

Leaves from trees grown under sunny conditions had higher TNC levels, irrespective of leaf age and girdling. Girdling increased TNC levels when trees were grown under sunny conditions, but not under shady conditions. Mature leaves had greater TNC than young leaves when trees were under sunny conditions. In contrast, young leaves had greater TNC content under shady conditions.

Girdling generally decreased P:C. The reduction in P:C was especially obvious in young leaves grown under shady conditions and in mature leaves grown under sunny conditions. Under sunny conditions, P:C was almost

Table 1 Leaf age, shade, and girdling of *Fraxinus pennsylvanica* on leaf chlorophyll content (mean±SEM μmol m^{-2})

Light	Control		Girdled[a]	
	Young[b]	Mature[c]	Young	Mature
Shady[d]	287.6±23.2	518.6±15.6	294.3±14.1	524.7±26.6
Sunny	323.3±28.7	499.5±17.0	221.9±14.4	428.6±24.8
Statistics				
Source	DF	F	P	
Light	1	6.37	< 0.05	
Wound	1	7.03	< 0.01	
Leaf age	1	196.71	< 0.01	
Light*Wound	1	9.47	< 0.01	
Light*Leaf age	1	1.70	> 0.05	
Wound*Leaf age	1	0.25	> 0.05	
Light*Wound*Leaf age	1	0.27	> 0.05	

[a] A 2.5 cm band of phloem removed from stem at a height of 15 cm from the soil

[b] Young and expanding leaves

[c] Mature leaves: ca. 3–4 weeks after full expansion, and not senescent

[d] Shade treatment was a 1.2×0.7×1.1 m (L×W×H) cage built with 1.9 cm (inner diam) PVC pipes covered with TapeKnit shade cloth. The shade cloth reduced the amount of sunlight available to the trees by approximately 70–80%.

doubled in young leaves from girdled trees compared to mature leaves from girdled trees. P:C of control trees under sunny conditions was not affected by leaf age. On the contrary, under shady conditions, mature leaves had greater P:C compared to young leaves, regardless whether trees were girdled or not. Shading elevated P:C by more than double compared to leaves from trees under sunny conditions, irrespective of leaf age and girdling.

Table 2 Foliar proteins, amino acids, and total non-structural carbohydrate (TNC) content (mean±SEM) of *Fraxinus pennsylvanica* seedlings under different treatments

Light			Proteins(mg g^{-1} FW[a])		Amino acids (μmol g^{-1} FW)		TNC (mg g^{-1} FW)		Protein:TNC ratio (w/w)	
	Wounding		Young[b]	Mature[c]	Young	Mature	Young	Mature	Young	Mature
Shady[d]	Control		13.7±1.4	14.8±0.7	6.6±1.0	16.9±1.6	11.9±1.2	8.2±0.7	1.3±0.2	1.9±0.2
	Girdled[e]		9.7±0.7	14.0±1.1	4.8±1.1	18.5±1.1	14.7±1.4	9.2±0.8	0.7±0.1	1.6±0.1
Sunny	Control		14.5±1.5	14.9±1.3	0.8±1.3	8.1±1.6	24.9±1.8	21.0±2.4	0.6±0.1	0.8±0.1
	Girdled		15.5±1.8	13.8±0.6	0.3±0.7	7.2±1.4	32.0±3.1	51.2±4.3	0.5±0.1	0.3±0.0
Statistics										
Source		*DF*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Light		1	3.63	> 0.05	33.49	< 0.01	39.15	< 0.01	25.80	< 0.01
Wound		1	2.05	> 0.05	0.21	> 0.05	41.13	< 0.01	33.50	< 0.01
Leaf age		1	1.50	> 0.05	128.98	< 0.01	0.95	> 0.05	8.55	< 0.01
Light*Wound		1	1.92	> 0.05	0.13	> 0.05	27.37	< 0.01	1.54	> 0.05
Light*Leaf age		1	4.03	< 0.05	8.61	< 0.01	14.63	< 0.01	25.08	< 0.01
Wound*Leaf age		1	0.11	> 0.05	0.90	> 0.05	11.20	< 0.01	2.18	> 0.05
Light*Wound*Leaf age		1	2.53	> 0.05	1.2	> 0.05	15.17	< 0.01	12.89	< 0.01

[a] fresh weight

[b] Young and expanding leaves

[c] Mature leaves: ca. 3–4 weeks after full expansion, and not senescent

[d] Shade treatment was a 1.2×0.7×1.1 m (L×W×H) cage built with 1.9 cm (inner diam) PVC pipes covered with TapeKnit shade cloth. The shade cloth reduced the amount of sunlight available to the trees by approximately 70–80%

[e] A 2.5 cm band of phloem removed from stem at a height of 15 cm from the soil.

Effects on Green Ash Foliage Defensive Compounds Trypsin and chymotrypsin inhibitors, and total phenolics of *F. pennsylvanica* foliage in response to light, girdling, and leaf age are shown in Table 3. The level of trypsin inhibitors was more than 30% greater in young leaves than in mature leaves. Light exposure and girdling had no significant influence on levels of trypsin inhibitors. The interaction between light and leaf age on chymotrypsin inhibitors was significant. Under sunny conditions, young leaves had more than three times higher chymotrypsin inhibitor levels, compared to mature leaves. However, under shady conditions, the difference in chymotrypsin inhibitors was not significant. Growth under sunny conditions more than doubled chymotrypsin inhibitor levels of young leaves, but had no significant effect in mature leaves.

The amount of total phenols in foliage from the sunny treatment was more than twice as high as in foliage from the shade treatment. Light exposure interacted with leaf age: under sunny conditions, young leaves had more than twice the level of total phenols compared to mature leaves; however, under shady conditions, mature leaves had greater total phenols than young leaves. Girdling decreased total phenolics of young leaves under shady conditions, while it elevated total phenolics of mature leaves under sunny conditions.

EAB Performance in Response to Green Ash Foliage Subject to Age, Shade, and Girdling Survival of EAB adults feeding on green ash foliage of various treatments over time is shown in Fig. 1A–C. Survival of EAB grown on mature leaves was greater than that on young leaves (Fig. 1A). There was no significant difference in survival of EAB feeding on foliage grown under sunny compared to shady conditions (Fig. 1B), or foliage from girdled vs. control trees (Fig. 1C). Similarly, only leaf age significantly influenced EAB adult longevity, which was greater on older leaves (Fig. 1D).

Discussion

Effects of Leaf Age on Plant Nutritional Quality and Defense Young and growing tissues generally are believed to contain higher quantities of nutrients (Mattson 1980; Harper 1989; White 1993), because these tissues require more resources to support their rapid growth. Total soluble protein accounted for less than 1.6% of the fresh weight of leaf tissues in this study, which might indicate the scarcity of nitrogen sources for arthropods feeding on green ash foliage. The amounts of total soluble protein did not differ between young and mature leaves (Table 2). However, mature leaves had greater levels of amino acids than young leaves, which is contrary to what is typically believed. Amino acid levels in plants and animals are in many cases

Table 3 Trypsin and chymotrypsin (mean±SEM) of *Fraxinus pennsylvanica* seedlings foliage under different treatments

Light		Trypsin inhibitors (mg g^{-1} FW[a])		Chymotrypsin inhibitors (mg g^{-1} FW)		Total phenolics (mmol g^{-1} FW)	
	Wounding	Young[b]	Mature[c]	Young	Mature	Young	Mature
Shady[d]	Control	15.5±1.7	14.5±2.8	2.3±0.4	2.7±0.3	17.4±2.7	21.2±1.5
	Girdled[e]	20.7±2.5	16.3±3.5	2.2±0.6	2.7±0.7	10.0±1.2	20.7±1.1
Sunny	Control	17.8±2.1	10.8±3.5	4.9±1.4	1.6±0.5	52.8±4.8	22.2±1.6
	Girdled	21.9±2.6	12.1±3.3	5.5±1.1	1.7±2.0	57.9±5.5	27.4±1.2
Source	*DF*	*F*	*P*	*F*	*P*	*F*	*P*
Light	1	0.05	> 0.05	1.56	> 0.05	0.00	> 0.05
Wound	1	2.87	> 0.05	0.04	> 0.05	2.39	> 0.05
Leaf age	1	9.46	< 0.01	4.77	< 0.05	11.89	< 0.01
Light*Wound	1	0.06	> 0.05	0.06	> 0.05	0.01	> 0.05
Light*Leaf age	1	2.45	> 0.05	7.57	< 0.01	5.45	< 0.05
Wound*Leaf age	1	0.74	> 0.05	0.02	> 0.05	0.64	> 0.05
Light*Wound*Leaf age	1	0.01	> 0.05	0.03	> 0.05	0.00	> 0.05

[a] fresh weight

[b] Young and expanding leaves

[c] Mature leaves: ca. 3–4 weeks after full expansion, and not senescent

[d] Shade treatment was a 1.2×0.7×1.1 m (L×W×H) cage built with 1.9 cm (inner diam) PVC pipes covered with TapeKnit shade cloth. The shade cloth reduced the amount of sunlight available to the trees by approximately 70–80%

[e] A 2.5 cm band of phloem removed from stem at a height of 15 cm from the soil.

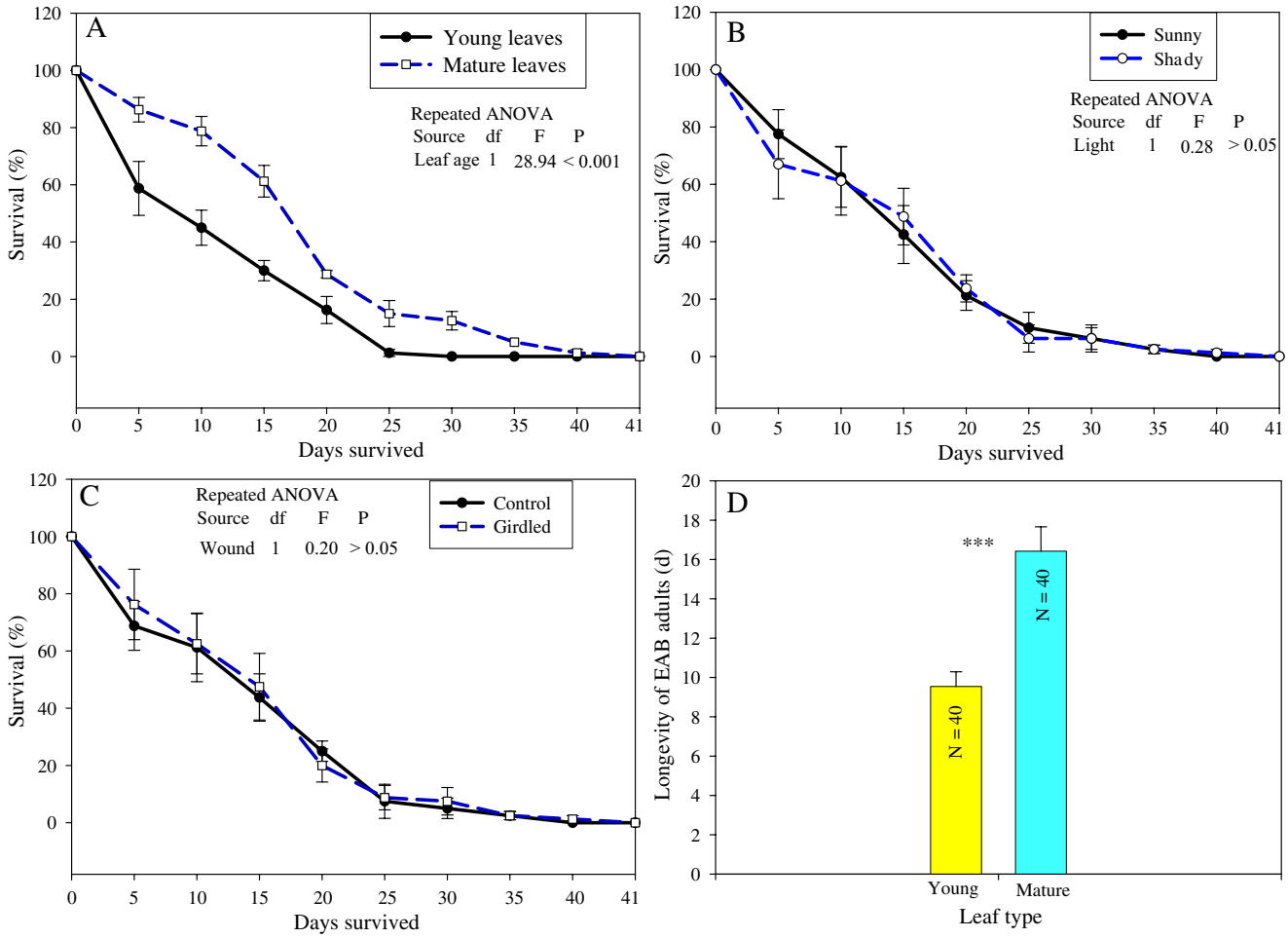

Fig. 1 Survival (means±SEM %) of Emerald Ash Borer (EAB) adults over time (**A**–**C**) and longevity (**D**). Newly emerged EAB were used for bioassay. Survival data were analyzed with repeated (time) ANOVA. Comparison was separately conducted between Young and Mature leave treatments, between Sunny and Shady treatments, and between Control and Girdled treatments. Young leaves: young and expanding leaves; Mature leaves: ca. 3–4 weeks after full expansion, and are not senescent. Standard errors of some data points do not appear because they are either smaller than symbols representing these data points or they are zero. *** $P<0.001$

elevated by stress (Good and Zaplachinski 1994; Bischof 1996; Dadmarz et al. 1998). We were not aware of any stress other than shade and girdling that the experimental seedlings experienced in this study. Therefore, higher amount of total amino acids in mature than young green ash leaves may be intrinsic. Nutrient balance, especially the ratio of protein to digestible carbohydrate (P:C) also has been shown to be important for the development of many insects; several of them chose higher P:C over lower P:C when given choices in the laboratory using artificial diets (Lee et al. 2002; Bede et al. 2007). The effects of leaf age on P:C interacted with the effect of light exposure: under sunny conditions, young leaves had greater P:C than mature leaves, especially in girdled trees, because young leaves had less TNC than mature leaves. On the other hand, under shady conditions mature leaves had higher P:C compared to young leaves, because older leaves had less TNC than young leaves. Interactive effects of leaf age and light condition on tannin concentration have been observed in *Inga oerstediana* seedlings (Nichols-Orians 1991). Thus, light condition may need to be considered when studying the effect of leaf age on nutritional qualities and defensive chemistry.

Young leaves had greater levels of trypsin and chymotrypsin inhibitors, and total phenolics than mature leaves in this study (Table 3). This is *consistent* with Optimal Defense theory, predicting that plant parts with higher fitness value should be better defended (McKey 1979). The greater fitness value of young expanding leaves over older leaves has been experimentally demonstrated in other study systems (McKey 1979; Strauss et al. 2004), as has the elevated accumulation of chemical defenses in these leaves (Ohnmeiss and Baldwin 2000; Chen et al. 2008b).

Effects of Light or Shade on Plant Nutritional Quality and Defense Exposure to sunlight elevated total proteins of

young green ash leaves, whereas it did not affect proteins of mature leaves in the study. The increased proteins in young leaves under sunny conditions might be partly attributable to trypsin and chymotrypsin inhibitors because the pattern of protein changes in young leaves under sunny conditions parallels that of trypsin and chymotrypsin inhibitor levels (discussed below). In contrast, exposure to sunlight decreased amino acid levles and P:C ratio, irrespective of leaf age and girdling. The lower P:C of leaves in the sun is due to a greater rate of increase in TNC in these leaves rather than an increase in total proteins. Although sunlight exposure reduced leaf chlorophyll levels (Table 1), it brought about net accumulation of TNC in foliage grown in the sun. On the other hand, although shading increased leaf chlorophyll content in compensation for lower sunlight, it brought about a net decline of TNC. Sunlight sharply elevated amounts of chymotrypsin inhibitors (nitrogen-based defensive chemicals) and total phenolics (carbon-based defensive compounds) of young leaves, but not mature leaves. A similar pattern of light effects on tannin levels of young and mature leaves has been observed in *Inga oerstediana* (Nichole-Orians 1991). The tannin levels of young *I. oerstediana* leaves grown to maturity in 20% of full sunlight was increased compared to young leaves grown to maturity in 2% of full sunlight. However, light intensity did not change tannin content of already mature leaves. The increase in chymotrypsin inhibitors for young leaves in the sun was *inconsistent* with the predictions of the CNB hypothesis that argues that trees grown under sunny environments should have lower nitrogen-based defensive but higher carbon-based defensive compounds (Bryant et al. 1983), while the elevation of total phenolics in young leaves in the sun followed the CNB predictions. The fact that defensive compounds, both carbon- and nitrogen-based, were elevated only in young leaves, highlights the need for caution when interpreting data without considering possible interactions such as leaf age. This also indicates the need to consider as many interactions as possible when developing hypotheses.

Effects of Girdling on Plant Nutritional Quality and Defense Girdling reduced leaf nutritional quality, in particular of young leaves grown in the shade and of mature leaves grown in the sun. The reduction of P:C was mainly due to accumulated TNC. Girdling is known to elevate TNC amounts above the girdled sites in many plant species including white ash, *F. Americana* (Noel 1970; Roper and Williams 1989; Li et al. 2003). The interactive effects of girdling, leaf age, and sunlight intensity on TNC indicated that the effects of girdling on foliage TNC were dependent on leaf age and growing conditions of seedlings. The greater photosynthetic capacity of mature leaves over young leaves grown in the sun (higher leaf chlorophyll levels; Table 1) might explain the accumulation of TNC in mature leaves from girdled trees in the sun. However, the increased accumulation of TNC in young leaves from girdled trees in the shade is contradictory to the higher photosynthetic capacity of mature leaves over young leaves from girdled trees grown in the shade, which is also indicated in Table 1. One explanation is that girdling makes TNC in young leaves grown in the shade more demanding, and TNC synthesized in mature leaves is exported to young leaves. No significant changes in protease inhibitors and total phenolics were observed following girdling. This is surprising since a variety of plant defensive chemicals are known to be induced after mechanical wounding and insect herbivory inflicted on leaves (Stout et al. 1998; Chen et al. 2008b). One possible explanation is that plants may respond differently to foliar damage and phloem injury.

EAB Adult Performance in Response to Green Ash Foliage Subjected to Various Treatments Increased EAB adult longevity was observed only on mature leaves compared to young and expanding leaves. This was probably attributable mainly to weaker defense (lower protease inhibitors and total phenolics) of mature leaves. Greater amino acids and P:C ratio of mature leaves, especially, higher P:C ratio in the shade, might also confer better performance of EAB adults on mature leaves. Reduction of light elevated amino acid and P:C ratio. However, EAB longevity was not affected by light intensity. A similar pattern was observed between girdled and control trees in that girdling decreased the nutritional quality of foliage, but EAB performance was not affected. The failure to detect EAB performance difference between low and high nutritional food might be explained by compensatory feeding of EAB adults that fed upon low quality foliage, since defensive compounds between these treatments were not significantly affected. Compensatory feeding of many herbivores on low quality host plants has been documented (Mattson 1980; Scriber and Slansky 1981).

In summary, leaf age, light exposure, and girdling interactively affected green ash nutritional and defensive compounds. Both nutritional quality and defensive compounds might contribute to EAB adult performance in this study and feeding preferences reported elsewhere (Poland et al. 2005; McCullough et al. 2006). However, the role of TNC in EAB preferences deserves special attention because the accumulation of TNC in foliage from girdled trees and trees grown in the sunny areas parallels EAB adult preference to these trees over corresponding un-girdled trees and trees grown in the shady areas. Additionally, although girdling and sun exposure altered nutritional and defensive chemistry of green ash foliage, EAB adult performance in the no-choice tests was not affected. The underlying mechanisms need further investigation. Eluci-

dation of these mechanisms contributes to ash tree breeding programs to develop resistant varieties or hybrids.

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The Influence of Eastern North American Autumnal Migrant Monarch Butterflies (*Danaus plexippus* L.) on Continuously Breeding Resident Monarch Populations in Southern Florida

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Abstract In Florida, the eastern North American population of the monarch butterfly exhibits geographic variability in population structure and dynamics. This includes the occurrence of migrants throughout the peninsula during the autumnal migration, occasional overwintering clusters that form along the Gulf Coast, remigrants from Mexico that breed in north-central Florida during the spring, and what have been assumed to be year-round, resident breeding populations in southern Florida. The work reported here focused on two monarch populations west of Miami and addressed four questions: Are there permanent resident populations of monarchs in southern Florida? Do these breed continuously throughout the year? Do they receive northern monarchs moving south during the autumn migration? Do they receive overwintered monarchs returning via Cuba or the Yucatan during the spring remigration from the Mexican overwintering area? Monthly collections and counts of spermatophores in the bursa copulatrices of females established that a resident population of continuously breeding monarchs exists year-round in southern Florida. It was determined through cardenolide fingerprinting that most of the butterflies had bred on the local southern Florida milkweed species, *Asclepias curassavica.* During the autumn migration period, however, some monarchs had fed on the northern milkweed, *Asclepias syriaca*. It appears that instead of migrating to Mexico, these individuals travel south through peninsular Florida, break diapause, mate with and become incorporated into the resident breeding populations. None of the monarchs captured in spring had the *A. syriaca* cardenolide fingerprint, which is evidence against the southern Florida populations receiving overwintered remigrants from Cuba, Central America or Mexico.

Keywords *Asclepias syriaca* · *A. curassavica* · Biogeography · Cardenolide · Thin layer chromatography fingerprints · Danainae · Migration · Milkweed · Population biology · Resident breeding populations · Everglades · Lepidoptera

Introduction

In North America, monarch butterflies *Danaus plexippus* L. (Nymphalidae) breed east of the Rocky Mountains to the Atlantic Coast and undergo a southward migration of up to 3,900 km from the northern United States and Canada to overwintering sites in central Mexico, followed by a spring remigration back into the U.S.A. of up to an additional 2,100 km (Brower et al. 2006). A smaller population breeds west of the Rockies and migrates to numerous overwintering sites along the California coast (Frey and Schaffner 2004). Monarchs also migrate in eastern Australia (review in James 1993), where they were introduced in the 1870s (Ackery and Vane-Wright 1984). In contrast, non-migratory, continuously breeding populations occur throughout the Neotropics and the West Indies (Urquhart 1960; Barcant 1970; Brower 1985) and in introduced populations on several Pacific islands and in parts of Australia (James 1993).

A long debated question that bears on the evolutionary exploitation of a temperate flora by an essentially tropical insect (Brower 1995; Ackery and Vane Wright 1984) is whether continuously breeding, non-migratory monarch

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butterfly populations occur in the southern United States. Reasons for this debate were Williams' (1958) contention that North American monarchs do not reproduce in their overwintering areas, and the subsequent confusion between overwintering and breeding populations reported from southern Texas (Urquhart 1960), southwestern Arizona (Funk 1968), southern California (Urquhart et al. 1970), and Florida (Urquhart 1960). Brower (1962) added to the debate by reporting what he considered local breeding populations during the spring in south-central Florida. Subsequent research determined that these populations were most likely a new spring generation established by remigrants returning from overwintering sites in Mexico (Knight 1998; Knight et al. 1999).

Limited evidence of breeding during the winter in southern Florida at one of the sites described in this study (Site 1) was presented by Brower (1985). He determined that 15 of 17 females collected on 5 December 1975 had mated, and also noted five mating pairs. Farrey and Davis (2004) reported monarch larvae on milkweeds growing in a southern Florida garden in January. The possibility exists then, that populations of monarchs in southern Florida may breed year-round. Other unresolved issues are the extent to which southern Florida monarch populations may be derived from the eastern North American migratory population, the possibility that they originated as immigrants from resident breeding populations in the West Indies, or that introgression may occur from both sources.

To resolve these issues, this study was carried out on two monarch populations in southern Florida immediately west of Miami, on the eastern edge of the Everglades. Four questions are addressed: Are the populations present throughout the year? Do they breed during all months of the year? Do they receive northern monarchs moving south during the autumn migration? Do they receive monarchs remigrating northward through the Florida peninsula during the spring?

To answer the first two questions, monarchs were censused nearly every month from September 1994 to November 1995 and their breeding status was determined by dissecting females and counting the number of spermatophores in their bursa copulatrices. The last two questions were addressed by analyzing chemical fingerprints of cardenolides present in individual monarchs collected from the two Miami populations throughout the same period. This technique utilized thin-layer chromatography (TLC) to visualize the arrays of cardenolides extracted from individual butterflies that reflect the species-specific pattern of these poisons that are present in the milkweed species (Asclepiadaceae) on which the monarch fed while a larva (Brower et al. 1982). Because different milkweed species have distinct thin-layer chromatography patterns, it is often possible to determine the general geographic area where a monarch adult was born, based on the milkweeds' discreet geographic distributions (Woodson 1954).

This method allows differentiation of autumn migrant monarchs that would have fed as larvae on northern milkweeds, predominantly *Asclepias syriaca* L. (Malcolm et al. 1993; Wassenaar and Hobson 1999), from monarchs that would have fed on southern Florida milkweeds, principally *A. curassavica* L. *Asclepias syriaca* is the most abundant eastern North American milkweed species and is distributed from southern Canada to Virginia (Woodson 1954). Because of human influence, it recently has extended its range to north central Georgia, but not Florida (Wyatt 1996). *Asclepias curassavica* occurs widely in the Neotropics and on the Caribbean islands (Woodson 1954). It is also abundant in cattle pastures in southeastern Florida (Cohen 1983, 1985). Several other species of milkweed occur in southern Florida and are discussed below.

Methods and Materials

Monarch Collections and Sampling Sites Adult monarchs were collected from two field sites near Miami, Florida. The butterflies were netted, placed in glassine envelopes, and kept continually on ice in a cooler, then frozen in a standard laboratory freezer until analyzed.

Site 1, the main collecting area was a ≈ three ha pasture in Hialeah, Florida (25°53′N, 80°22′W) and surrounded a water filled limestone quarry. The pasture contained large patches of *A. curassavica* as well as two other less abundant milkweeds, *A. incarnata* Walt. and *Sarcostemma clausum* (Jacq.) Schult. The site was bordered to the south by a stand of large live oaks, *Quercus virginiana* L. (Fagaceae), in which monarchs were observed roosting and as resting mated pairs. *Sida* sp. (Malvaceae) also grew in the pasture and was a potential nectar source. Censuses were made in March–April 1990 and monthly from September 1994 to November 1995, except for November 1994 and June and August 1995. Reference to Google Earth, "Graham Baptist Church", access date 9 May 2009, indicates that the old field vegetation surrounding the quarry was destroyed, probably around 2002.

Site 2 (25°48′N, 80°20′W) was ≈ three ha area 10 km south of Site 1. It consisted of a fenced cattle pasture containing abundant but scattered *A. curassavica* plants, and a larger, ungrazed old-field that contained a few *A. curassavica* and a variety of composites (Asteraceae) that served as nectar sources. Monarchs were monitored from February to May 1995, and one sample was collected here for thin-layer chromatography analyses on 7 Nov 1995.

Wing Condition and Mating Status Wing condition for each butterfly was rated visually from 1 (fresh, virtually no scales missing) to 5 (worn, many scales missing) in increments of 0.5. Mating frequency was determined for

all females collected in 1994 and 1995 by dissecting their bursa copulatrices and counting spermatophores, following the method of Van Hook (1999). Butterflies collected in 1990 were too desiccated for bursa copulatrix dissections.

Chemical Procedures In order to perform TLC analyses and determine the foodplants that the butterflies had fed upon as larvae, butterflies were assayed for cardenolide contents as follows. They were dried at 60°C for 16 h in a forced draft oven and weighed on a Mettler AK 160 balance (Mettler-Toledo, Inc., Columbus, OH, USA). Following Alonso-Mejia et al. (1997), each dried butterfly was ground in petroleum ether to extract the lipids. Following Malcolm et al. (1989), cardenolides were ethanol-extracted from the individually defatted butterflies in 10 ml volumetric flasks and spectroassayed to determine gross concentration as µg/0.1 g of dry butterfly material. A Lambda IIs dual beam spectrophotometer (Perkin Elmer Corporation, Norwalk, CT, USA) was used for all spectroassays.

Seven ml of each remaining ethanol extract were cleaned of plant pigments for TLC in order to visualize the component cardenolides by using the tetranitrodiphenyl (TNDP) reagent (Brower et al. 1982; Malcolm et al. 1989; Moranz and Brower 1998). Chromatography was performed on the extracts of a total of 408 monarchs. Each TLC plate was spotted with 14 monarch extracts along with 10 µg of digitoxin and digitoxigenin cardenolide standards spotted on the center and sides of each plate (Fig. 1a and b). Following visualization, each plate was photographed on Kodachrome 25 film, and the images were transferred digitally to a compact disc. Adobe Photoshop (ver. 2.0) was used to sort individual butterflies by cardenolide pattern, as described in Moranz and Brower (1998).

Discriminating Cardenolide Fingerprints of Monarch Adults that Fed as Larvae on Various Milkweed Species The various fingerprints were sorted by comparing the cardenolide spot intensities and mobilities with published fingerprints of the monarch and/or queen butterfly (*Danaus gilippus* Cramer) reared on several milkweed species. The two most critical fingerprints are those found in monarchs that fed as larvae either on the southern Florida *A. curassavica* (Fig. 1a and b) or the northern *A. syriaca* (Fig. 1b). Published *A. curassavica* fingerprints are in Cohen 1983; Brower 1984; Malcolm et al. 1989; Knight 1998, while published *A. syriaca* fingerprints are in Brower 1984; Seiber et al. 1986; Malcolm et al. 1989; Knight 1998. Several other species of milkweeds occur in central to

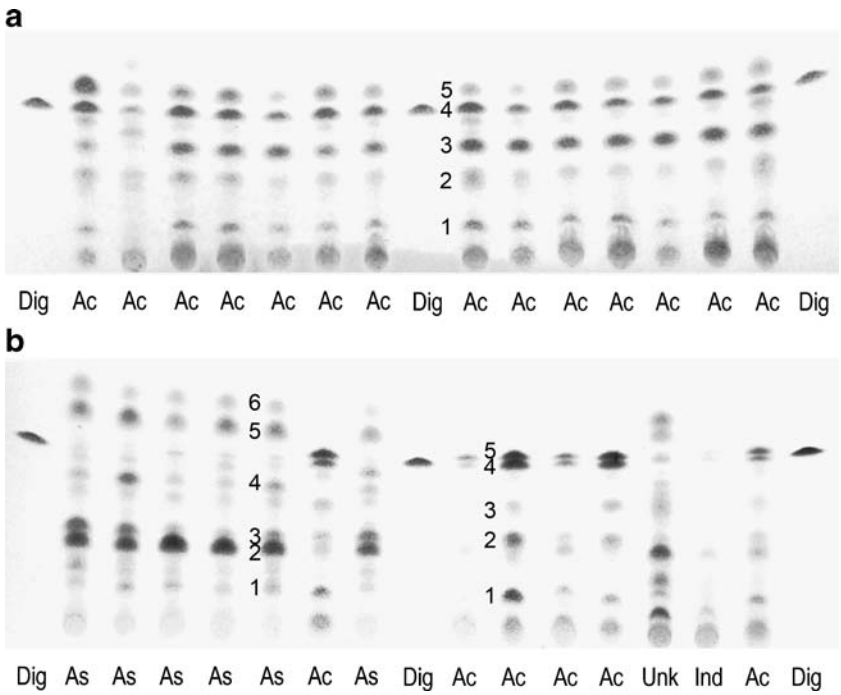

Fig. 1 **a** Thin-layer chromatogram plate of 14 monarchs captured in southern Florida at Site 1 on 1 January 1995, with 10 µg of digitoxin (Dig) standards. The butterflies were all females from which 75 µg of extracted cardenolide were applied to the single plate. Unnumbered spots are not cardenolides. All butterflies have the *Asclepias curassavica* (Ac) cardenolide fingerprint. **b** Thin-layer chromatogram plate of 14 monarchs captured in southern Florida at Site 2 on 7 November 1995, with 10 µg of digitoxin (Dig) standards. Unnumbered spots are not cardenolides. From left to right, the first three channels are from males, and the rest from females; only 7 µg were available for nos. 8 and 10, 3 µg for no. 13, and 32 µg for no. 14; the applied amount for the rest of the channels was 75 µg. Six butterflies exhibit the *Asclepias curassavica* (Ac) cardenolide fingerprint, seven the *A. syriaca* (As) fingerprint, one butterfly is *indeterminate* (Ind), and one has an as yet unknown (Unk) fingerprint

southern Florida (Woodson 1954). These include *A. perennis* Walt., *A. longifolia* Michx., *A. viridis* Walt., *A. humistrata* Walt., *A. lanceolata* Walt., and three vinaceous milkweeds, *Matelea gonocarpa* Walt. (Shinners), *Cynanchum scoparium* Nutt., and *C. angustifiolium* Pers. All of these produce monarchs and/or queens with fingerprints that are qualitatively distinct from monarchs that feed on *A. curassavica* or *A. syriaca* (Martin et al. 1992; Moranz and Brower 1998). Two other common Florida species are *A. incarnata* L. with only small amounts of cardenolide, and *A. tuberosa* L. that has virtually none (Malcolm 1991; Brower, Seiber, and Nelson, unpublished data). Both produce faint to no monarch fingerprints that are distinct from *A. curassavica* and *A. syriaca*. Cardenolide fingerprints have not been determined for three other southern Florida milkweeds: *A. verticillata* L., *A. pedicellata* Walt., and the vine, *Sacrostemma clausum* (Jacq.) Schult. The first two are relatively uncommon, and *A. pedicellata* is Florida's smallest milkweed that has little foliage to support a monarch larva. It is probable that *Sarcostemma*, like *Cynanchum* and *Matelea* (Moranz and Brower 1998), has similar weak fingerprints, vividly distinct from both *A. syriaca* and *A. curassavica.*

The monarch fingerprints were designated as indeterminate when there was an insufficient amount of cardenolide on the TLC plate to determine a clear pattern, or as unknown when a pattern was clear but could not be matched to a known one.

Results

Cardenolide Fingerprints Thin-layer chromatography revealed that at least 84% of the 379 adult monarchs collected in 12 of the 13 months at Site 1 had the *A. curassavica* cardenolide fingerprint, with 100% having this fingerprint in six of the months (Table 1; Fig. 1). In November 1995, eight of 11 individuals (73%) had the *A. curassavica* fingerprint, and three (27%) had the *A. syriaca* fingerprint. In total, 11 monarchs from Site 1 had fed as larvae on *A. syriaca*. Significantly, all *A. syriaca* monarchs were collected in autumn: six in October 1994, three in November 1995, and one each in December 1994 and October 1995. Three had fingerprints that were indeterminate, and two had unknown fingerprints.

TLC results for the Site 2 collection of 29 monarchs on 7 November 1995 had a higher proportion of *A. syriaca* monarchs (58%) than did the collection made at Site 1 one day earlier (28%); Table 1; Fig. 1). Excluding the two indeterminate individuals and the one unknown, this difference is significant (Site 2, 17/26=65%; Site 1, 3/11=27%; $\chi_1^2=4.90$, $P<0.05$). The indeterminate monarchs probably had also fed on *A. syriaca* plants that had low cardenolide concentrations. Monarchs that feed on this milkweed vary from low to high concentrations both in migrants (Knight 1998) and in those collected from across the northern summer breeding range (Malcolm et al. 1989). Overall, only eight of the 408 monarchs analyzed could not be fingerprinted to a known milkweed species; 91% had fed on *A. curassavica*, and 7% on *A. syriaca*.

Frequency of Female Mating Of the 139 females dissected from Site 1, 133 (96%) had mated (Table 2). Five of the six virgins had excellent wing condition (1 or 1.5), suggesting that they had bred locally, and that enough time had not elapsed since eclosion for them to have mated. Of the 133

Table 1 Summary of thin-layer chromatography fingerprints of 408 adult monarch butterflies collected in March and April, 1990 and from September 1994 through November 1995 from Site 1, and in November 1995 at Site 2

Site	Date	Year	N	Butterfly cardenolide fingerprint			
				Indet[a]	Unknown[b]	A. syriaca	A. curassavica
1	19 Mar-17 Apr	1990	46	0	0	0	46 100%
1	8 Sep	1994	26	0	0	0	26 100%
1	22 Oct	1994	38	0	0	6	32 84%
1	17 Dec	1994	45	0	1	1	43 95%
1	1 Jan	1995	41	1	0	0	40 98%
1	10 Feb	1995	35	0	0	0	35 100%
1	12 Mar	1995	28	0	1	0	27 96%
1	8 Apr	1995	31	0	0	0	31 100%
1	8–9 May	1995	39	0	0	0	39 100%
1	20 Jul	1995	10	0	0	0	10 100%
1	10 Sep	1995	19	2	0	0	17 90%
1	26 Oct	1995	10	0	0	1	9 90%
1	6 Nov	1995	11	0	0	3	8 73%
2	7 Nov	1995	29	2	1	17	9 31%
Total			408	5 (1.2%)	3 (0.7%)	28 (6.9%)	372 (91.2%)

[a] Fingerprint is indeterminate

[b] Fingerprint is distinct but unknown

Table 2 Mating frequency determined by spermatophore counts for 155 female monarchs collected in 1994 and 1995 from Site 1, and in November 1995 from Site 2

Site	Date	Year	N	Numbers of spermatophores per bursa						
				Mean	Range	0	1	≥2	% mated	% multiply-mated
1	8 Sep	1994	7	2.4	1–5	0	3	4	100	57
1	22 Oct	1994	21	2.8	1–5	0	3	18	100	86
1	17 Dec	1994	24	2.7	0–8	1	3	20	96	83
1	1 Jan	1995	25	4.3	0–10	2	3	20	92	80
1	10 Feb	1995	11	5.5	2–9	0	0	11	100	100
1	12 Mar	1995	10	4.3	1–9	0	1	9	100	90
1	8 Apr	1995	13	3.3	1–7	0	2	11	100	85
1	8–9 May	1995	6	2.2	0–4	2	0	4	67	67
1	20 Jul	1995	6	2.8	1–4	0	1	5	100	83
1	10 Sep	1995	6	3.0	0–6	1	1	4	83	67
1	26 Oct	1995	5	2.6	1–4	0	1	4	100	80
1	6 Nov	1995	5	2.4	1–4	0	2	3	100	60
2	7 Nov	1995	16	2.9	0–10	2	5	9	88	56
Total			155	3.3	0–10	8	25	122	95	79

females that had mated, 113 (85%) were multiply mated, with a maximum of ten spermatophores counted in one individual. The mean number of spermatophores ranged from 2.2 in May 1995 to 5.5 in February 1995. The high frequency of multiple matings in all months indicates that mating occurs throughout the year in this population.

Of the nine *A. syriaca* females collected at Site 1, all had mated, and seven (78%) had multiply mated. These northern migrants had thus broken diapause and must be exchanging genes with the Miami population. Mating frequency also was high for the 16 females collected at Site 2, with 88% mated and 56% multiply mated. Twelve of these 16 females (75%) had the *A. syriaca* cardenolide fingerprint, ten (84%) were mated, and six (50%) were multiply mated. The two unmated females had wing conditions of 2.0, suggesting that they may have been recently arrived migrants from the north that still were in reproductive diapause.

Wing Condition Mean wing condition for all monarchs captured was 2.6 (SD=1.0). Monarchs with excellent (1.0 or 1.5) wing condition were captured in every month sampled except November 1995, Site 1, where the best condition was 2.0.

Discussion

This study addressed whether resident monarch populations occur in southern Florida, and, if so, whether they breed continuously through the year. Monthly collections confirmed that two populations on the eastern edge of the Everglades near Miami occur year-round, and periodic research at one of these sites indicates that monarchs as well as abundant *A. curassavica* and *A. incarnata* plants have occurred there for at least 20 years, in December 1975 (Brower 1985), December 1981 (Cohen 1983), September 1984 (Malcolm and Brower 1986), March–April 1990 (this paper), and September 1994–November 1995 (this paper).

Monarchs were collected in every month visited from September 1994 to November 1995. Although no visits were made in June and August of 1995, the population appeared stable throughout the study, as indicated by the numbers of monarchs captured during each visit. The capture and bursa dissection results, along with the constant presence of *A. curassavica* as a food plant and the *A. curassavica* cardenolide fingerprint in the butterflies collected at both sites are evidence for a viable, year-round continuously breeding resident monarch population in this part of southern Florida.

Bursa copulatrix dissections of female monarchs confirmed that the monarchs breed year-round. Sixty-seven to 100% of the females were mated in all months, with most multiply mated. Similarly, Brower (1985; unpublished data) found that 15 of 17 females captured at Site 1 on 5 December 1975 had mated from two to seven times, saw four mating pairs, and also determined that two fresh females had not mated. These results are similar to those found in resident populations of monarchs in Trinidad, West Indies, where mating frequency in the summer was 96%, with 52% multiply mated (Pliske 1973), and for summer breeders both in Massachusetts, U.S.A. and in Australia where 98% of the females were mated, with 80 and 95% multiply mated, respectively (Brower 1985; Suzuki and Zalucki 1986). Finally, the wing condition also provided evidence that new generations are being produced

throughout the year: at least some individuals in each non-autumn month had wing conditions of 1.0 or 1.5, indicating recent eclosion.

Year-round breeding of monarchs may not be possible throughout much of the southeastern U.S. because of warm summer temperatures. Based on the laboratory findings of Zalucki (1982) and Rawlins and Lederhouse (1981) that constant temperatures between 31 and 35.5°C are lethal for monarch larvae, Malcolm et al. (1987) deduced that high early summer temperatures in the Gulf coastal states force the new spring generation produced during late March and April to move north, which they do in early May (Knight et al. 1999). It seems likely, however, that maximum summer temperatures in the Miami area are slightly below this threshold most of the time. The mean monthly maximum temperature for 30 years (1961–1990) from April to September at the Miami airport was 31.7°C (Anon. 1999). Further north in Florida, however, temperatures may exceed the threshold sufficiently often to be lethal. For example, in Gainesville approximately 480 km north of Miami, the mean monthly maximum temperature for 30 years (1961–1990) from April to September was 32.6°C (Anon. 1999).

In contrast, warm temperatures are required for monarchs to breed year-round and thus sustain a population in southern Florida because neither the butterflies nor their larval food plants can survive severe freezing temperatures (Ackery and Vane-Wright 1984; Larsen and Lee 1994; Anderson and Brower 1996) that periodically affect north Florida and the Gulf Coast (Brower 1995). Although warm temperatures also occur throughout the winter in the southwestern United States, where winter breeding has been reported, these areas probably cannot sustain a permanent year-round breeding population due to the dieback of milkweeds during the autumn and winter (Funk 1968; Urquhart et al. 1970). This is not the case, however, in southern Florida, where the climate is sufficiently warm and wet to sustain year-round populations of *A. curassavica*, a nearly ubiquitous neotropical milkweed (Woodson 1954) that is the principal food plant of monarchs throughout the West Indies, Central and South America (Barcant 1970; Haber 1993). Thus, the combination of warm temperatures and a constant host plant supply, which promotes year-round reproduction in southern Florida, resembles conditions in the tropics where continuously breeding, resident monarch populations occur widely.

This study also addressed whether southern Florida populations receive migratory monarchs from the north during the autumn. The TLC results for October and November 1994 and 1995 show clearly that the southern Florida resident population receives an influx of autumnal migrant monarchs that have fed on the northern milkweed, *A. syriaca*. Moreover, 90% of these *A. syriaca* females were mated or multiply mated, indicating that they had broken reproductive diapause and were becoming incorporated into the resident southern Florida population. The two unmated females appeared to be recent arrivals still in reproductive diapause, which is characteristic of most autumn migrants along the Florida Gulf coast and at the overwintering sites in Mexico (Herman et al. 1989, Van Hook 1999). Mating frequencies of autumnal migrants along the northern Florida Gulf Coast have been observed at 29% (Brower 1985) and 38% (A. Knight and L. P. Brower, unpublished), which are much lower than those reported here for the migrants at the southern Florida sites.

Environmental factors strongly influence reproductive activity in monarchs. Based on physiological experiments, Barker and Herman (1976) proposed that monarchs in the Gulf coast states remain in reproductive arrest as long as average temperatures are below 28°C. Along with the evidence from the present study, this suggests that the migrants must become reproductively active when they reach the warmer, southern areas such as southern Florida and the West Indies (Urquhart 1960; Brower 1985). The incorporation of these autumnal migrants into the southern Florida population is further indicated by the fingerprint results that no monarchs that had fed on *A. syriaca* were present in March and April, 1990 or in January to September, 1995.

The final question addressed by this study is whether there is evidence for a spring remigration of monarchs that had overwintered in Cuba or the Yucatan into these southern Florida populations, as originally postulated by Urquhart and Urquhart (1976). The lack of the *A. syriaca* fingerprint in any of the southern Florida samples collected during March through May argues against this hypothesis. *A. syriaca* monarchs do appear regularly each spring in north-central Florida, but these are likely individuals returning from the Mexican overwintering sites. As far as is known, all Caribbean and southern Florida monarch populations are resident and non-migratory, and the possibility that they migrate northward is not supported by a long-term study of migration patterns of Florida butterflies (Walker 2001), nor by any evidence cited in reviews of the migration biology of the monarch butterfly (Brower 1985, 1995).

Research by Dockx et al. (2004) on monarchs collected in Cuba is in agreement. These authors combined cardenolide fingerprinting with stable isotope analyses and determined that 62% of 152 monarchs that were collected at three Cuban sites during November in 1993, 1995, 1996, and 1997 had migrated there from southeastern Canada or the northeastern United States. Of these, 18 were determined to have fed as larvae on *A. syriaca.* As in the southern Florida samples, no *A. syriaca* butterflies were found in a March sample.

The evidence from both studies provides support for the hypothesis (Brower 1985, 1995) that autumn migrants

flying south through the Florida peninsula are off the normal course to their overwintering area in central Mexico. These individuals, in effect, become physiologically trapped, break reproductive diapause, and, rather than remigrating northward the following spring, they and their offspring become assimilated into the resident breeding populations.

In summary, monthly collections confirmed that monarchs on the eastern edge of the Everglades occur year-round, and bursa copulatrix dissections of females indicated that the population is continuously reproductive. Cardenolide fingerprinting revealed that, except in the autumn, virtually all monarchs had fed as larvae on *A. curassavica*, a neotropical milkweed that grows extensively in southern Florida but does not occur naturally in the monarch's northern range. Taken together, the findings provide evidence for a year-round breeding population and for its persistence through the years.

Cardenolide fingerprints of *A. syriaca* present in butterflies collected during October–December established that northern migrants enter the Miami population during the autumn. Moreover, 90% of these *A. syriaca* females were mated or multiply mated, indicating that they had broken reproductive diapause and were becoming incorporated into the resident southern Florida population. The lack of the *A. syriaca* fingerprint in any of the spring butterflies is evidence against a spring remigration of northeastern monarchs that have migrated to and overwintered in Cuba or the Yucatan back into these southern Florida populations.

Finally, although the cardenolide fingerprints in our samples were distinct and their interpretation straightforward, multivariate analysis of fingerprint patterns could further confirm these results. We propose to conduct High Performance Liquid Chromatography (HPLC) by using remaining cardenolide extracts from these samples and to develop a statistical interpretation method.

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Stigmasterol and Cholesterol Regulate the Expression of Elicitin Genes in *Phytophthora sojae*

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Abstract Sterol acquisition by soilborne plant pathogens of the genus *Phytophthora* is presumed to involve extracellular proteins belonging to class-I elicitins. However, little is known about the relationship between sterol availability and elicitin secretion. The objective of this study was to determine the expression of class-I elicitin genes in *Phytophthora sojae* when grown in a medium containing stigmasterol or cholesterol. *P. sojae* growth was stimulated by nanomolar concentrations of stigmasterol and cholesterol, which also resulted in the down-regulation of its elicitin genes over time when expression profiles were monitored using real time Reverse Transcription Polymerase Chain Reaction (RT-PCR). The down-regulation of elicitin genes in response to the two sterols also coincided with a reduction in the amount of elicitins detected in spent filtrates. Our study is the first to show the influence of sterols on elicitin gene expression in *Phytophthora*, which is important with respect to the ecology of elicitin secretion as sterol carrier proteins in the environment.

Keywords Elicitin · Extracellular proteins · *Phytophthora* · Plant pathogens · Sterol carrier protein

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Introduction

Oomycetes are a diverse group of eukaryotic organisms that include the genus *Phytophthora*, many species of which are destructive plant pathogens in agricultural and forest ecosystems (van West et al. 2003). Members of *Phytophthora* all lack sterol biosynthetic pathways and acquire sterols exogenously from their environment to support their growth and sexual reproduction (Hendrix 1975; Elliot and Knights 1981; Nes and Stafford 1983; Ponchet et al. 1999; Marshall et al. 2001). It is speculated that these organisms acquire sterols from the environment via extracellular proteins belonging to class-I of elicitins (Ponchet et al. 1999). These are small (10 kDa) hydrophilic secreted proteins that were classified as sterol carrier proteins because some exhibited sterol and lipid carrier activity *in vitro* (Mikes et al. 1997, 1998; Vauthrin et al. 1999; Osman et al. 2001a, b).

Elicitins are proteins unique to the genus *Phytophthora,,* and are encoded by a large multi-gene family that is divided into at least eight different classes based on sequence homology and protein motif diversity (Jiang et al. 2006a, b). Class-I elicitins are the best characterized, and are considered structurally simple when compared to other elicitin classes (Jiang et al. 2006b). They encode only a conserved 98 amino acid sequence (known as the elicitin domain) and a signal peptide that is post translationally removed (Ponchet et al. 1999; Jiang et al. 2006b), whereas other classes may also encode a variable c-terminal domain that may contain a membrane-spanning domain (Jiang et al. 2006a, b). Elicitin genes are highly conserved across the entire genus of *Phytophthora* (Kamoun et al. 1997a, b; Qutob et al. 2003; Jiang et al. 2006a, b); all species of *Phytophthora* studied so far secrete elicitins abundantly into

their growth medium (Ponchet et al. 1999), suggesting a significant role for elicitins in the biology of these organisms, presumably in sterol uptake.

Class I elicitins became of interest when it was shown that these proteins induced a plant hypersensitive response (HR) when infiltrated on tobacco leaves (Ponchet et al. 1999). Consequently, further research was done to establish whether elicitin genes are avirulence genes that determine host range (Ponchet et al. 1999; Kamoun 2001). As a result, studies on elicitins have focused largely on elicitor activity on plants (e.g., Baillieul et al. 2003), characterization of protein structure (Lascombe et al. 2004), and discovery of new elicitins secreted by *Phytophthora* spp. (Churngchow and Rattarasarn 2000). However, there are no studies on the significance of elicitin secretion with respect to sterol availability and *Phytophthora* ecology. In soil, sterols are described as labile organic compounds (Bull et al. 2000); *in vitro* studies show that *Phytophthora* spp. assimilate exogenously supplied sterols into their membranes (Elliot and Knights 1981), but the relationship between sterol availability and elicitin secretion has not been established. Therefore, the objective of this study was to examine the expression profile of class-I elicitin genes in the model oomycete and pathogen of soybean, *Phytophthora sojae*, when two sterols of different origin, stigmasterol (plant) and cholesterol (animal), are supplemented in its growth medium.

Methods and Materials

The *Phytophthora sojae* culture (race 1) was obtained from Dr. Dorrance (OARDC, Wooster OH, USA). Cultures were maintained on non-clarified V8 agar (18% v/v V8 agar juice, 0.3% $CaCO_3$, and 2% agar) at 25°C in the dark. For growth response bioassays and gene expression experiments, plugs (6 mm or 10 mm diam) were transferred from the edge of actively growing *P. sojae* into a defined minimal medium (Wu et al. 2003) modified to contain glucose (10 g/l). The growth medium was solidified with 2% w/v agar (growth response experiments) or kept in liquid state (gene expression experiments). Cultures were incubated in the dark at 25°C until terminated.

Biological Assays Stigmasterol and cholesterol (95–99% purity) (Sigma-Aldrich, St. Louis, MO USA) were added to the growth medium at a final concentration of 1,000 nM, 100 nM, or 10 nM with chloroform (final 0.01% v/v) as the solvent. For growth response bioassays, single plugs (6 mm diam) were transferred from the edge of actively growing *P. sojae* on V8 agar to the center of experimental agar plates, and incubated in the dark at 25°C. Colony diameter was measured over time with a metric ruler. For gene expression and protein profile experiments, single plugs (10 mm diam) were inoculated into Erlenmeyer flasks (50 ml) that contained experimental media (20 ml). *P. sojae* mycelia were harvested over time by vacuum filtration through pre-weighted nylon membranes (0.2 μm), and replicates were either processed immediately for RNA extraction or dried in an oven overnight (45°C) for dry biomass measurements. All experiments were repeated independently twice. Experiments included three biological replicates. For gene expression experiments, RT-PCR data was generated from three technical replicates of two biological replicates.

Primer Specificity for Elicitin Amplification P. sojae contains at least nine elicitin genes (Qutob et al. 2003), two of which are class-I and named *SOJA* and *SOJB* (referred to as *sojein* in Mao and Tyler 1996 and Becker et al. 2000). The letters A and B following *SOJ* further classify the elicitins according to their isoelectric (pI) points as either acidic (A, pI<5) or basic (B, pI>5), depending on the total number of Lys residues present in the elicitin domain (Ponchet et al. 1999; Qutob et al. 2003). Moreover, four isotypes of the *SOJ*A gene are present, (originally named sojein 1–4 in Mao and Tyler 1996 and Becker et al. 2000) that differ from each other by a few amino acid substitutions within the elicitin domain, which results in a slightly different amino acid content. Primers for the *SOJA* and *SOJB* elicitin genes were designed from their cDNA sequences available on the National Center for Biotechnology Information (NCBI) web-site (Table 1), whereas primers for the internal control Actin gene (*ActA*) were designed from sequences of the gene available for *P. sojae* on the US Department of Energy's Joint Genome Initiative (JGI) web-site (Table 1).

Designing primers that are specific for each individual *SOJA* isotype is difficult because differences in the nucleotide sequences are minor. Instead, we designed primers to regions that are homologous to the elicitin domain in all four *SOJA* isotypes, which would amplify a 298 bp fragment of the expressed genes. Elicitin genes lack introns (Mao and Tyler 1996; Ponchet et al. 1999), which allowed us to use genomic DNA from *P. sojae* as a template to test the specificity our elicitin primers. The elicitin domain of *SOJA* is highly conserved and contains nucleotide sequences that are similar to *SOJB* and other elicitin classes present in *P. sojae*. To determine if our *SOJA* primers would amplify other elictin genes having homologous sequences, we cloned the PCR products into *E.coli* and submitted ten individual colonies selected at random for sequencing. Using BLAST, nine of the sequenced clones were identified as *Sojein2* (*SOJA-2*), and one was identified as *Sojein3* (*SOJA-3*). For *SOJB*, primers were designed to amplify a 120 bp fragment of the

Table 1 Oligonucleotide primers used in real-time RT-PCR expression analysis and amplicon size

Gene	GenBank accession	Amplicon size (bp)	Forward and reverse primers
SOJA	AJ007858 (*sojein1*), AJ007859 (*sojein2*), AJ007860 (*sojein3*), AJ007861 (*sojein4*)	298	5′ acc acg tgc acc tcg tcg cag 3′ 5′ tta cag cga cgc gca cgt gga 3′
SOJB	AY183409	120	5′tct aag cgc gtc ctc cag ctc 3′ 5′ctc aca act tag tcc tcg gtt gat ggc 3′
ActA	estExt_fgenesh1_pm.c_490003[a]	264	5′gta ctg caa cat cgt gct gtc g 3′ 5′ tta gaa gca ctt gcg gtg cac g 3′

[a] Expressed sequence tag available from the US Department of Energy's Joint Genome Initiative (JGI)

3′untranslated region (UTR) of the gene because the 3′ UTR region is reported to be variable in elicitin genes (Jiang et al. 2006a, b).

Cloning of SOJA Elicitin Genes The *SOJA* elicitin genes were amplified from genomic DNA of *P. sojae* by regular PCR using the following primers; 5′ATCGAATTC ACCACGTGCACCTCG 3′ (forward) and 5′ AAT CTC GAG TTA CAG CGA CGC GCA CGT GGA CGA GAA 3′ (reverse). These primers are similar to the ones used for gene expression assays, but include *Eco*RI (forward primer) and *Xho*I (reverse primer) sites for cloning and sequencing to confirm the specificity of amplification. The underlined sequences correspond to the elicitin gene flanked by a restriction enzyme site. The cycle conditions were 94°C for 2 min, followed by 35 cycles of 92°C for 1 min, 56°C for 1 min, 72°C for 30 s, and a final extension at 72°C for 5 min. The amplicons were cut with *Eco*RI and *Xho*I, purified on 1% agarose, and ligated into a PGEX-4T-1 vector (GE Health Care, Piscataway, NJ, USA). *E. coli* DH5α was transformed with the construct, and ten individual clones from each amplification reaction were sequenced at the Robarts Research Sequencing facility (The University of Western Ontario, London, ON, Canada).

RNA Extraction and cDNA Synthesis RNA was extracted from mycelia of *P. sojae* using the RNeasy Plant Mini Kit (Qiagen cat # 74904) according to the manufacturer's protocol. The concentration of RNA was determined by using the Quant-iT ^{Tm} Ribogreen RNA assay kit (Invitrogen cat# R11490) via a NanoDrop ND-3300 Fluorospectrometer. Equivalent amounts of starting RNA (approximately 0.3 μg) from each sample were used as template for first strand cDNA synthesis with the QuantiTect Reverse transcription kit (Qiagen cat# 205311). A preliminary step to remove any contaminating genomic DNA prior to cDNA synthesis is included in the QuantiTect Reverse transcription kit.

RT-PCR Amplification and Analysis Amplification of cDNA was carried out in a final volume of 25 μl that contained 3 μl of a 10-fold dilution of the first strand cDNA, and 0.25 μM primers (*SOJA*) or 0.5 μM primers (*SOJB* or *ActA*) and 12.5 μl iQ™ SYBR Green super mix (Bio-Rad cat# 170-88805). Reactions were performed with a iQ5 multicolor real-time thermal cycler (iQ™ 5 optical module, Bio-Rad, Hercules, CA, USA) for 2 min at 94°C followed by 40 cycles of 94°C for 40 s, annealing at 67°C (*SOJ*A and *SOJ*B) or 59°C (*Act*A) for 40 s, and extension at 72°C for 30 s. The $2^{-\Delta\Delta C}$T method for relative quantification of gene expression was validated as described in (Livak and Schmittgen 2001) prior to the evaluation of elicitin gene expression. Untreated controls (*P. sojae* only receiving delivery solvent) served as calibrators for the treatments, whereas *Act* A served as an internal control to normalize for any variation in the amount of RNA that was initially added to the reverse transcription reaction. Amplification of specific transcripts was confirmed by melting curve analysis provided by the LightCycler instrument and length of PCR products on agarose gels (1.5%). The amplified products also were confirmed at least once by sequencing at the Plant Microbe Genetics facility (The Ohio State University, Columbus, OH, USA).

Protein Analysis The Bradford protein assay (Bradford 1976) was used to quantify the total amount of protein in the spent filtrates of *P. sojae* using γ-globulin as standard. Spent filtrates from replicates of each treatment subsequently were pooled into one sample, and each pooled sample (15 μl) was analyzed on 4–12% gradient Bis-Tris sodium dodecyl sulfate polyacrylamide gel (Invitrogen cat# NP0322BOX) with MES as the running buffer (Invitrogen cat# NP0002) using the XCell SureLock^{Tm} Mini cell system (Invitrogen, Carlsbad, CA, USA) at 200 V. Protein gels were silver stained with the SilverXpress silver staining kit (Invitrogen cat# LCG100) according to the manufacturer's protocol.

For 2D-PAGE analysis, proteins present in 5 ml of spent filtrates from day 13 of controls were precipitated from solution with four volumes of cold acetone (−20°C) according to a protocol published in a technical resource (PIERCE -TR00490.0, Rockford, IL, USA) and dissolved in 4.4% (w/v) CHAPS (200 µl) and urea solution (8 M). The entire volume was used to rehydrate a 3–10 pH IPG strip via a passive rehydration step (1 h) followed by an active rehydration step at 50 V (14 h). The rehydration step was followed by focusing the proteins on the IPG strip overnight at 60,000 vhr. Following focusing, the strips were equilibrated in urea (6 M), Tris buffer (0.375 M; pH8.8 for 25 min), and then in SDS(2% w/v), glycerol (20% w/v), DTT (2% w/v) for 25 min. The equilibrated IPG strips subsequently were run onto pre-cast criterion gels (10–20%) in TGS buffer at 200 V for 60 min, then fixed in methanol (10%) and acetic acid (7%) for 30 min prior to being stained overnight with Sypro Ruby (Bio-Rad cat# 170-3126) according to the manufacturers protocol. Three putative elicitin bands were excised from the gel, placed into water and submitted to the Mass Spectrometry and Proteomics Facilty (The Ohio State University, Columbus, OH, USA) for digestion and analysis by capillary-liquid chromatography-nanospray tandem mass spectrometry (Nano-LC/MS/MS) prior to identification using MASCOT (Matrix Science version 2.2.1, Boston, MA, USA), a search engine that uses MS data to identify proteins from primary sequence databases such as NCBI.

Statistical Analysis Colony diameter measurements, gene expression assay, biomass measurements, and extracellular protein assays from experiments were analyzed by factorial *ANOVA* followed by a *Bonferroni* or *LSD pairwise* comparison of means (α=0.05) using Statistix™ analytical software (P.O. Box 12185, Tallahassee, FL, USA).

Results

Response of P. sojae Sterols Stigmasterol is structurally similar to cholesterol, differing only by the presence of an additional 24-ethyl group and a *trans*-22-double bond (Fig. 1). The sensitivity of *P. sojae* to different concentrations of stigmasterol and cholesterol was determined by dose response growth assays (Fig. 2). Although the pathogen did grow in the absence of sterols, supplementation of growth medium with sterols enhanced the growth rate, as evident by an enhanced stimulation of its mycelium (Fig. 2).

Concentrations of stigmasterol or cholesterol ranging from 1 µM to 1,000 µM stimulated *P. sojae* mycelia equally well (data not shown). However, we were interested in determining the lowest concentration threshold required to stimulate *P. sojae* growth. Interestingly, *P. sojae* appears to be 10-fold more sensitive to stigmasterol than to cholesterol, as 10 nM of stigmasterol was the lowest concentration required to cause a significant (P<0.001) growth difference compared to controls (Fig. 2) vs. 100 nM needed for cholesterol to cause a similar response (Fig. 2).

Transcripts for both *SOJA* and *SOJB* elicitin genes were detected from cDNA generated from *P. sojae* mRNA, indicating both isoforms were expressed by the organism (Fig. 3). This was also confirmed by 2D-PAGE analysis of spent filtrates from control cultures (Fig. 4), which showed three protein bands with a molecular weight of approximately 10 kDa and pI's in the range of 3, 6, and 9. These proteins were confirmed to be elicitins of *P. sojae* after tryptic digestion and analysis of the individual bands using LC/ MS-MS) (data not shown).

By using a dilution series of cDNA from control cultures, we optimized PCR conditions to obtain equivalent amplification efficiencies for the target genes and the internal control gene used to normalize treatments. RT-PCR conditions for amplification of the targets (*SOJA* and *SOJB*) and internal reference gene (*ActA*) were optimized to yield respective amplification efficiencies of 104, 94, and 99% for the *SOJA, SOJB,* and *ActA* transcripts respectively. The amplification efficiency values for target genes fall within approximately 5% of the E value of the internal reference gene. This allowed the use of the $2_T^{-\Delta\Delta C}$ method (Livak and Schmittgen 2001) for the relative expression analysis of elicitin genes. Running the qPCR products on a 1.5% agarose gel showed the presence of a single band at approximately 300 bp, 250 bp, and 100 bp for *SOJ*A, *Act*A, and *SOJ*B, respectively (data not shown). The identity of the qPCR bands was confirmed by sequencing, and the characteristic melting temperatures for *SOJ*A, *SOJ*B, and *Act*A qPCR products were 90.5°C, 80.5°C, and 88.5°C, respectively.

Elicitin Gene Expression in Response to Stigmasterol and Cholesterol The class-I elicitin genes, *SOJA and SOJB*, are constitutively expressed in *P. sojae*, as transcripts for these genes were present in sterol untreated control samples. However, supplementing the growth medium of *P. sojae* with cholesterol or stigmasterol resulted in a differential expression profile of class-I elicitin genes compared to control samples. Generally, class-I elicitin genes became significantly (P<0.001) down-regulated over time compared to untreated controls (Fig. 3), but there appears to be a temporal delay in the down-regulation of *SOJA* and *SOJB* elicitin genes in response to cholesterol when compared to stigmasterol (Fig. 3). Specifically, elicitin genes became significantly (P<0.001) down-

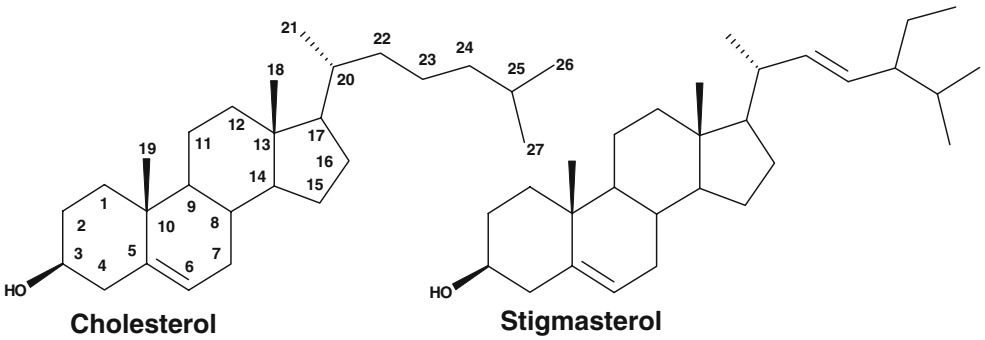

Fig. 1 Chemical structure of the sterols used to supplement *Phytophthora sojae* growth medium. Both sterols are structurally similar except that stigmasterol contains a trans-22 double bond and a 24-ethyl group in the side chain

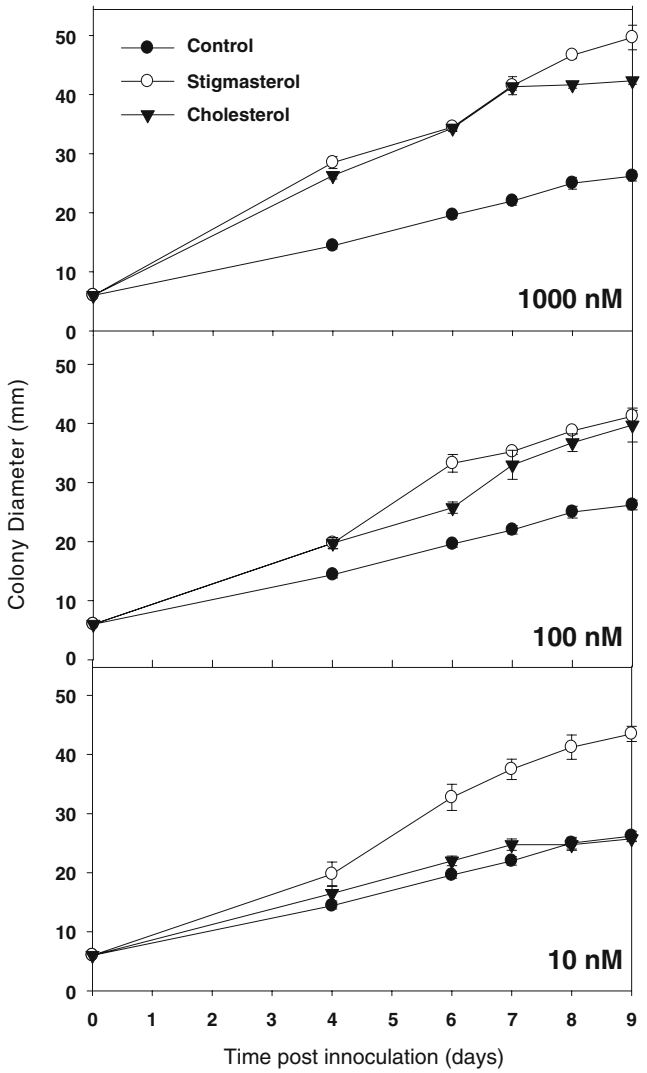

Fig. 2 *Phytophthora sojae* growth measurement (hyphal extension) over time after its growth medium was supplemented with a log-based gradient concentration of 1,000 nM, 100 nM, or 10 nM of either stigmasterol or cholesterol. Data plots represent three replicates ± SE from one experiment. ANOVA followed by a bonferroni pair-wise comparison of means identified significant differences (α=0.05, $P<$ 0.001) in colony diameter measurements between the 10 nM stigmasterol treatment and control, and between the 10 nM stigmasterol and 10 nM cholesterol treatments. No significant difference was identified between the 10 nM cholesterol treatment and the control

regulated on day-9 in response to stigmasterol (Fig. 3), whereas a similar magnitude in elicitin down-regulation occurred on day-13 for cholesterol (Fig. 3).

Expression of class-I elicitin genes appears to be controlled by both negative and positive feedback mechanisms that are dependent on environmental sterol concentrations. An example of negative feedback that is dependent on sterol concentration is observed in the *SOJA* expression profile in response to stigmasterol (Fig. 3). On day-3, *SOJA* genes were down-regulated significantly in response to 1,000 nM of stigmasterol, whereas the down-regulation event was delayed and only observed on day-9 for the lower stigmasterol concentrations of 10 nM and 100 nM (Fig. 3). An example of positive feedback is observed on day-3 in the *SOJA* and *SOJB* expression profile in response to cholesterol (Fig. 3), and *SOJB* expression in response to stigmasterol (Fig. 3), in which the genes were significantly ($P<0.001$) up-regulated compared to untreated controls.

Extracellular Proteins in Spent Filtrates of P. sojae The total amount of secreted proteins in relation to dry biomass of *P. sojae* was determined over time (Table 2). Extracellular protein profiles were examined by 1D-PAGE analysis (Fig. 5). The goal was to determine if the down-regulation in *SOJA* and *SOJB* genes in response to sterols coincided with a reduced amount of secreted elicitin proteins in the spent filtrates of *P. sojae*. Elicitins are reported to be the most abundant proteins in filtrates of *Phytophthora* spp. (Ponchet et al. 1999). This is consistent with our results for *P. sojae*, as the only protein detected in spent filtrates was a single 10 kDa band that was confirmed as an elicitin of *P. sojae* by LC/MS-MS.

The amount of protein in the spent filtrates of control cultures continued to increase and reached a maximum concentration of 5 μg ml^{-1} on Day-13 (Table 2). The detection limit of the silver stain kit is 0.7 ng $band^{-1}$. Therefore, the concentration of proteins in spent filtrates prior to day 13 (1–2 μg ml^{-1}) was likely below the detection limit of the silver stain. The 10 kDa band detected from control filtrates on Day-13 cultures was absent in spent filtrates of the 1,000 nM cholesterol and stigmasterol

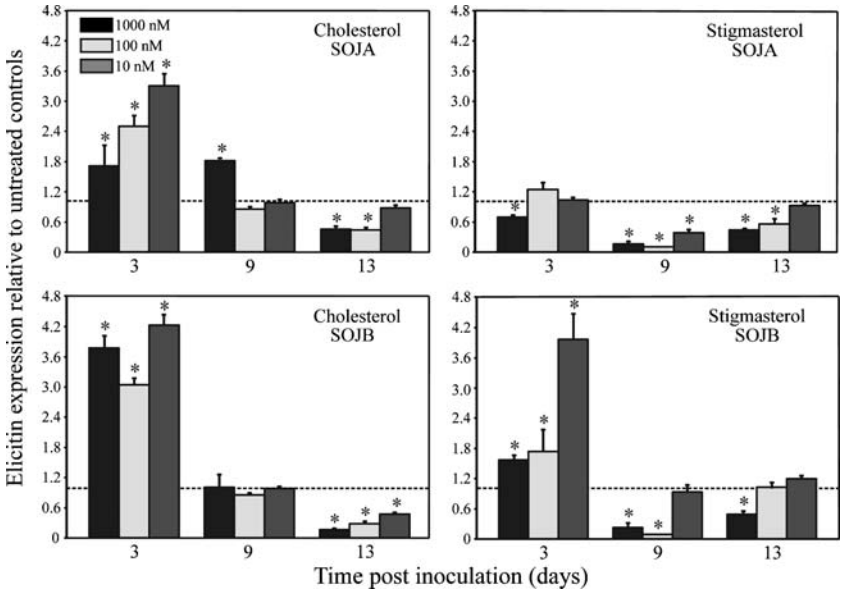

Fig. 3 *Phytophthora sojae* expression of class-I elicitin *SOJA and SOJB* genes was monitored over time in response to a log-based concentration gradient of 1,000–10 nM of cholesterol or stigmasterol using real-time RT-PCR. The y-axis shows expression relative to untreated controls (*P. sojae* not receiving sterol) after normalization to the internal control gene *Act*A(a value of 1.0 indicates no difference between treatment and control, a value greater than 1.0 indicates up-regulation of elicitin genes, and a value less than 1.0 indicates a down-regulation of elicitin genes). Bar graphs represent means of three technical replicates obtained from two biological replicates ± SE from two independent experiments. Bar graphs with a (*) indicate values significantly different from untreated controls after ANOVA followed by an LSD pair-wise comparison of means (α=0.05, P<0.001)

treatments (Fig. 5a) even though there was approximately 1.5 times more biomass in the same volume of experimental media compared to controls (Table 2). The reduced amount of proteins in the sterol treated samples (Fig. 5a) is supported by an analysis of the total amount of protein produced per biomass of *P. sojae* (Table 2). On Day-13, only 0.2 μg mg^{-1} protein was produced in the presence of 1,000 nM stigmasterol and 1.1 μg mg^{-1} for the same concentration of cholesterol compared to a higher amount (i.e., 4.2 μg mg^{-1}) produced by control cultures (Table 2).

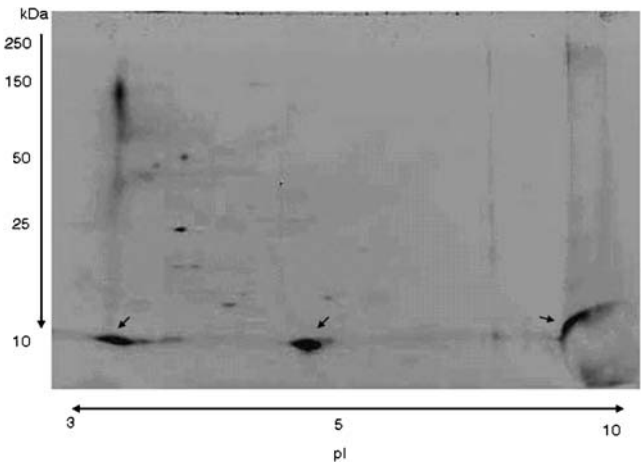

Fig. 4 Two-dimensional SDS-PAGE analysis of *Phytophthora sojae* extracellular proteins (25 μg) present in filtrates after 13 days of growth in a minimal medium in the absence of sterols. Proteins were separated according to their pI (x-axis) and size (y-axis). Arrows point to three putative elicitin isoforms with approximate pI values of 3, 5, and 9, and a molecular weight of 10 kDa. The identity of the proteins was later confirmed to be *P. sojae* elicitins by LC/MS/MS

The 10 kDa elicitin band was not detected in *P. sojae* spent filtrates treated with stigmasterol over the entire concentration range of 1,000 nM to 10 nM (Fig. 5), whereas it was detected when a cholesterol concentration of 100 nM (Fig. 5b) or 10 nM was used (Fig. 5c). Moreover, the staining intensity of the 10 kDa protein band reached levels similar to controls at the lowest cholesterol concentration (i.e., 10 nM) (Fig. 5c). This also is supported by the lack of differences in the amount of proteins produced per biomass between control cultures and those treated with 10 nM cholesterol (Table 2). This observation is in agreement with the biological dose response assays we conducted that showed the growth of *P. sojae* is more sensitive to stigmasterol compared to cholesterol (Fig. 2).

Discussion

Phytophthora sojae is a hemibiotroph that can survive in the soil as a saprotroph or as a heterotroph in the soybean host (Tyler 2007). In soil, the concentration of sterols varies between 0.2 ppm to 30 ppm and depends on the presence of

Table 2 Total proteins in spent filtrates of *phytophthora sojae* in response to stigmasterol and cholesterol over time

Treatment	Biomass (mg)	Secreted protein ($\mu g ml^{-1}$)	Protein/Biomass** ($\mu g mg^{-1}$)
Sample Day 3			
Control	6.5 a	1.1	3.4 a
Stigmasterol 1,000 nM	9.4 b	ND*	ND
Stigmasterol 100 nM	7.7 a,b	ND	ND
Stigmasterol 10 nM	8.1 a,b	ND	ND
Cholesterol 1,000 nM	7.7 a,b	ND	ND
Cholesterol 100 nM	7.3 a,b	ND	ND
Cholesterol 10 nM	6.9 a,b	0.5	1.2 b
Sample Day 9			
Control	12.3 a	2.1	3.4 a
Stigmasterol 1,000 nM	18.4 b	ND	ND
Stigmasterol 100 nM	16.5 b	1.4	1.7 a
Stigmasterol 10 nM	17.5 b	1.9	2.3 a
Cholesterol 1,000 nM	17.1 b	ND	ND
Cholesterol 100 nM	18.4 b	2.1	2.3 a
Cholesterol 10 nM	15.4 a,b	2.5	3.3 a
Sample Day 13			
Control	24.3 a	5.0	4.2 a
Stigmasterol 1,000 nM	43.2 b	0.4	0.2 b
Stigmasterol 100 nM	34.1 b	2.3	1.4 b,c
Stigmasterol 10 nM	32.9 b	2.9	1.8 b,c
Cholesterol 1,000 nM	29.2 a,b	1.6	1.1 b,c,d
Cholesterol 100 nM	30.4 a,b	3.3	2.2 c,d
Cholesterol 10 nM	26.6 a	4.3	3.3 a,d

Values followed by the same letter are not significantly different from each other using ANOVA followed by a Bonferroni comparison of means ($P< 0.05$)

*ND indicates no protein detected

**Protein per biomass was calculated according to 20 ml of total experimental volume

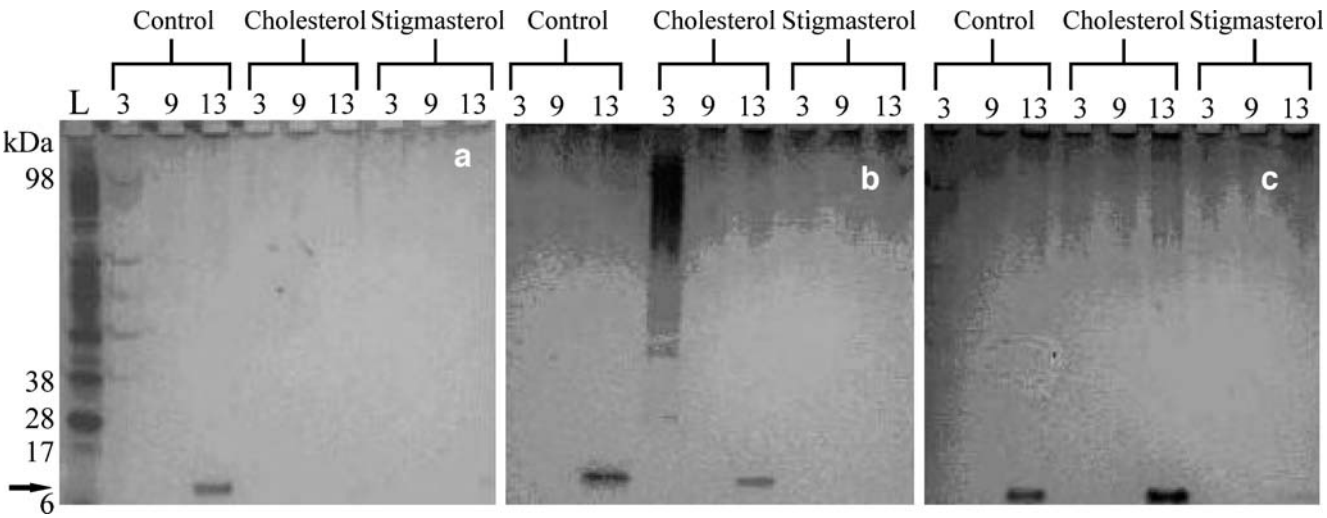

Fig. 5 Extracellular proteins present in filtrates of *Phytophthora sojae* grown in a minimal medium after 3 days, 9 days, or 13 days of growth in the absence of sterol (control), in the presence of 1,000 nM (**A**), 100 nM (**B**), or 10 nM (**C**) of either cholesterol or stigmasterol. The letter L shows standard protein markers with an approximate size range of 98 kDa to 6 kDa (SeeBlue Plus2 pre-stained standard, Invitrogen), whereas the numbers 3, 9, and 13 indicate days post inoculation of *P. sojae* into the experimental media. Spent filtrates (15 μl) containing a maximum amount of 75 ng total protein (e.g., control day 13) were separated on a 4–12% MES-SDS-PAGE gel and stained with silver nitrate. The arrows point to putative elicitin bands with a molecular size of approximately 10 kDa

soil organisms, plant matter, and organic fertilizer inputs (Puglisi et al. 2003). In soybean, sterol concentrations depend on the physiological stage of plant development, and vary between approximately 0.0002–0.005% of plant weight (Fenner et al. 1986). In this study, we evaluated mycelial extension and class-I elicitin gene expression of *P. sojae* in response to varying concentrations of stigmasterol and cholesterol. Mycelial extension of *P. sojae* was stimulated at nanomolar concentrations in response to both sterols, but was 10-times more sensitive to stigmasterol than to cholesterol. These results are significant and show that *P. sojae* (1) is exquisitely sensitive to the presence of sterols, responding to them at very low concentrations, and (2) apparently can discriminate between sterols with small structural differences. Our results are different from other sterol studies with *Phytophthora* (Wood and Gottlieb 1978; Nes and Stafford 1983; Marshall et al. 2001) because we utilized sterol supplement concentrations that are approximately 250 or 2,500 times lower than the lowest concentration of sterol supplements used in other studies to examine a physiological response. The ability of *P. sojae* to respond to sterols at nanomolar concentrations indicates that it is efficient in utilizing sterols, even at low concentrations in the environment.

Studies involving *P. parasitica* (pepper pathogen) (Colas et al. 2001) and *P. infestans* (potato pathogen) (Kamoun et al. 1997b) reported that the expression of elicitin genes in these organisms was down-regulated during plant infection. Colas et al. (2001) and Kamoun et al. (1997b) did not consider sterols as potential regulators of elicitin gene expression in *P. parasitica* or *P. infestans*. In our study, class-I elicitin gene expression in *P. sojae* appears to be controlled by both negative and positive feedback mechanisms that are dependent on sterol concentration and structure. Generally, a reduction in elicitin biosynthesis was observed when either stigmasterol or cholesterol was included in the growth medium of *P. sojae*. However, differences in the expression profile of *SOJA* and *SOJB* elicitin genes was observed in response to each sterol, which may be related to different binding efficiencies for these two sterols with elicitins encoded by the *SOJA* and *SOJB* genes.

Overall, cholesterol was less effective than stigmasterol in down-regulating elicitin expression since the kinetics of down-regulation by cholesterol were slower (Fig. 3). Moreover, the up-regulation of class-I elicitin genes in response to cholesterol on day-3 (Fig. 3) may have occurred to compensate for the slow kinetics of any of (1) elicitin binding with cholesterol, (2) elicitin-sterol complex binding to receptors on *P. sojae* membranes, or (3) metabolism of sterol once incorporated into cells. Following the same argument, the up-regulation of *SOJB,* but not *SOJA* elicitin genes, in response to stigmasterol may have occurred because the basic class-I elicitins encoded by the *SOJB* gene are less efficient in stigmasterol uptake than the acidic class-I elicitin proteins. A different study that involved the soilborne pathogen *P. cactorum* showed that the organism incorporated sitosterol into its mycelium more rapidly than it did cholesterol when a mixture of these two sterols was present in the growth medium (Elliot and Knights 1981), i.e., a mechanism involving elicitins maybe at play. Interestingly, a common observation made between this study and that of Elliot and Knights (1981) is both *P. sojae* and *P. cactorum* are plant pathogens that appear to process plant sterols (stigmasterol and sitosterol) more efficiently over cholesterol —a predominantly animal sterol. It maybe possible that *Phytophthora* plant pathogens have evolved to preferentially utilize sterols commonly present in their host.

In summary, our study shows that class-I elicitin genes of *P. sojae* are regulated by nanomolar concentrations of sterols, and that an apparent differential in temporal gene regulation occurs in response to small structural differences in sterol type (stigmasterol vs. cholesterol). Nonetheless, these results indicate that elicitins play a role in sterol uptake in *P. sojae*. It remains to be determined whether sterols act as diffusible factors that connect elicitin genes to the environment, or whether down-stream mechanisms with putative *P. sojae* elicitin receptors on membranes are involved.

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The Effects of Arbuscular Mycorrhizal Fungi on Direct and Indirect Defense Metabolites of *Plantago lanceolata* L.

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Abstract Arbuscular mycorrhizal fungi can strongly influence the metabolism of their host plant, but their effect on plant defense mechanisms has not yet been thoroughly investigated. We studied how the principal direct defenses (iridoid glycosides) and indirect defenses (volatile organic compounds) of *Plantago lanceolata* L. are affected by insect herbivory and mechanical wounding. Volatile compounds were collected and quantified from mycorrhizal and non-mycorrhizal *P. lanceolata* plants that underwent three different treatments: 1) insect herbivory, 2) mechanical wounding, or 3) no damage. The iridoids aucubin and catalpol were extracted and quantified from the same plants. Emission of terpenoid volatiles was significantly higher after insect herbivory than after the other treatments. However, herbivore-damaged mycorrhizal plants emitted lower amounts of sesquiterpenes, but not monoterpenes, than herbivore-damaged non-mycorrhizal plants. In contrast, mycorrhizal infection increased the emission of the green leaf volatile (*Z*)-3-hexenyl acetate in untreated control plants, making it comparable to emission from mechanically wounded or herbivore-damaged plants whether or not they had mycorrhizal associates. Neither mycorrhization nor treatment had any influence on the levels of iridoid glycosides. Thus, mycorrhizal infection did not have any effect on the levels of direct defense compounds measured in *P. lanceolata*. However, the large decline in herbivore-induced sesquiterpene emission may have important implications for the indirect defense potential of this species.

Keywords Arbuscular mycorrhizal fungi (AMF) · Volatile organic compounds (VOC) · Iridoid glycosides (IG) · *Plantago lanceolata* · *Spodoptera littoralis* · *Glomus intraradices* · Plant secondary metabolites

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Introduction

About 80% of all terrestrial plants form associations with arbuscular mycorrhizal fungi (AMF) (Wang and Qiu 2006). These are fungal symbionts that are well known to improve plant nutritional status by enhancing the uptake of essential nutrients such as phosphorous and nitrogen and by improving the water supply through an increase in root surface area (Smith and Read 1997). In return, fungi receive carbon in the form of photosynthates from the plant.

For both the establishment and the maintenance of the symbiotic association between plants and AMF, it is essential that both partners recognize each other. These recognition processes are initiated *via* molecular cross-talk mediated by changes in the gene expression and production of signal compounds in both partners (Harrison 2005). On the plant side, altered gene expression in the presence of AMF can influence other aspects of metabolism and even result in the induction of chemical defenses (Gange et al. 2007). A number of studies in the recent past have reported the effects of AMF on plant defensive compounds, such as volatile terpenoids (Akiyama and Hayashi 2002; Rapparini et al. 2008), essential oils (Copetta et al. 2006; Khaosaad et al. 2006) and glucosinolates (Vierheilig et al. 2000).

Furthermore, effects of AMF on salicylic acid (SA) and jasmonate dependent signaling pathways have been reported, suggesting that AMF modulate plant defenses (review by Pozo and Azcòn-Aguilar 2007 and references therein). AMF invasion triggers a general plant response to pathogen attack (Dumas-Gaudot et al. 2000), causing a transient accumulation of SA and activation of the SA-dependent signaling pathway at the early stages of the association. These responses then seem to be repressed once the compatibility of the symbionts is recognized (reviewed by Garcìa-Garrido and Ocampo 2002).

Two major forms of plant anti-herbivore defenses can be distinguished: direct defenses, which are toxic to the herbivore or deter feeding, and indirect defenses, which protect the plant by attracting natural enemies of the herbivore, either parasitoids or predators. Direct and indirect defenses can be constitutively expressed or induced by mechanical damage or herbivore feeding. The literature on mycorrhizal influence on direct and indirect defenses and the consequences for insect herbivores is scarce (Gange et al. 2007; Hartley and Gange 2009), although some studies that show the effects of AMF on direct defenses have been published (e.g., Marak et al. 2002; Fuchs and Bowers 2004). A major group of indirect plant defenses are volatile organic compounds (VOCs) that consist principally of green leaf volatiles (GLVs) and mono- and sesquiterpenes (Pichersky and Gershenzon 2002; Degenhardt et al. 2003). Herbivore-induced VOCs play an important role in attracting natural enemies of insect herbivores (e.g., Dicke et al. 1990; Turlings et al. 1990; Kessler and Baldwin 2001). Furthermore, herbivore-induced VOCs act as both intra- and inter- plant signals, and can result in priming and induction of plant defenses (e.g. Kost and Heil 2006; Frost et al. 2007; Heil and Silva Bueno 2007).

Naturally occurring VOC emissions have been compared in mycorrhizal vs. non-mycorrhizal plants. For example, mycorrhization of *Artemisia annua* L. with two AMF species did not affect the amount of total terpenes emitted, but there were slight changes in the relative quantities of single compounds (Rapparini et al. 2008). In addition, an unspecialized fungal root endophyte (*Acremonium strictum*) reduced terpene emission of tomato plants with consequences for insect oviposition preference (Jallow et al. 2008). However, no study to date has investigated how herbivore damage alters the production of defenses in AMF vs. non-AMF plants, even though herbivory is known to have marked effects on VOC emission profiles and the levels of other defense compounds.

Plantago lanceolata L. is a perennial forb with a cosmopolitan distribution and commonly forms associations with a large number of AMF species (Johnson et al. 2004; Oehl et al. 2004). The main group of secondary metabolites in *P. lanceolata* is the iridoid glycosides, with two dominant compounds, namely aucubin and catalpol. These compounds function as feeding and oviposition stimulants for specialized insects, and as deterrents or toxins for generalist herbivores (e.g., Bowers and Puttick 1988, Biere et al. 2004). Antimicrobial functions of these monoterpene derivatives also have been documented (Marak et al. 2002). The association of *P. lanceolata* with AMF can modify plant defense properties. In a study by Gange and West, the levels of the two iridoid glycosides (IGs), aucubin and catalpol, increased when the plants were associated with AMF (Gange and West 1994). However, the effects of AMF on other groups of defensive compounds in *P. lanceolata*, such as green leaf volatiles or volatile terpenoids, have not yet been documented.

In this study, we investigated the effects of the arbuscular mycorrhizal fungus *Glomus intraradices* (N.C. Schenck & G.S. Sm.) on *P. lanceolata* by focusing on two groups of compounds, IGs and VOCs, that typically act as direct and indirect plant defenses, respectively. In an experiment with a full factorial design, we compared VOC emissions and the IG contents of AMF-inoculated and non-inoculated *P. lanceolata* individuals after mechanical wounding and caterpillar herbivory with those of non-treated control plants.

Mycorrhizal fungi could influence a plant's allocation to defense in different ways: 1) *Altering nutritional status of the host plant:* Greater nutrient availability could lead to an increase in primary productivity that provides more resources for the plant to use in the biosynthesis of defensive metabolites, such as IGs or VOCs. On the other hand, harboring AMF is no guarantee of increased productivity, since in return for nutrients, plants provide symbiotic fungi with photosynthates. If the outflow of photosynthates to the fungal symbionts is greater than the increase in productivity due to enhanced nutrient supply, there may be a net decrease in carbon supply that could lead to a decline in defense metabolism. This decline might affect the production of direct vs. indirect defenses differently depending on the relative value of these defensive strategies under different nutritional conditions. 2) *Altering signalling pathways:* Independent of plant nutritional status, the presence of microorganisms, including AMF, could alter defense signalling. Microbial infection generally is known to activate many types of defense responses, although mycorrhizal fungi usually elicit only attenuated responses (Garcìa-Garrido and Ocampo 2002).

Methods and Materials

Plant, Fungus and Insect Material Seeds of *P. lanceolata* (Rieger & Hofmann, Germany) were sown in trays filled with commercially available sowing soil (Stender

Vermehrungssubstrat A210, Stender, Germany) that was previously autoclaved for 20 min at 121°C, in order to kill potential AMF propagules. *P. lanceolata* germinated and grew in a greenhouse (day:night temperatures 20–22°C: 18–20°C, 30–55% humidity, 16 h light, photosynthetically-active radiation ca. 180 µmol $m^{-2}s^{-1}$).

To prepare a growing medium, soil from a meadow in proximity to the greenhouse (in Jena, Germany) was mixed with sand in a 1:1 (w w^{-1}) proportion, and autoclaved for 20 min at 121°C. Thereafter, 178 pots (14.4 cm diam, 1.3 l) were each filled with 300 ml of the soil-sand mixture and watered with 10 ml soil suspension (500 g fresh soil suspended in 5 l tap water, filtered through a 25 µm Whatman filter to exclude AMF propagules) in order to allow the establishment of a new microbial community in the sterile soil mixture (Schroeder and Janos 2004). Afterwards, all pots were covered with gauze to prevent the soil from desiccation.

Glomus intraradices inoculum was purchased from Amykor (Germany) (strain AMYKOR1), and half of it was autoclaved for 30 min at 121°C. Half of the 178 pots were then inoculated with 5 g of AMF vital inoculum each ("mycorrhizal plants"), the other half were mock inoculated with the same quantity of AMF autoclaved inoculum ("non-mycorrhizal" plants). The inoculum was mixed with the upper layer (3–4 cm) of the soil-sand mixture, and the *P. lanceolata* seedlings were transplanted individually into the pots.

Due to a thrips infestation, all plants were treated once with Conserve (Dow AgroSciences LLC, USA) (Conserve 0.075 %, 0.5 l spray for all the 178 plants) 5 wk after transplantation.

Caterpillars of the generalist feeder *Spodoptera littoralis* Boisd. (Lepidoptera: Noctuidae) (Syngenta, Basel, Switzerland), were reared on an artificial bean diet at 21°C. The diet was prepared by mixing 1 l tap water with 1 kg beans, 18 g ascorbic acid, 10 g 4-ethylbenzoic acid, 18 g α-tocopherol (7.1% in germ oil), 8 ml 3.7% formaldehyde, and 2.4 l of a 5% agar solution) at 21°C. Third-instar caterpillars were starved for 24 h before they were used in the experiment.

Experimental Setup and Plant Treatments Plants were divided between mycorrhizal and non- mycorrhizal treatments, and 15 plants of a single treatment were placed in one tray (60×40 cm). The trays were positioned on a greenhouse bench in two rows, one with mycorrhizal plants and the other without, in order to avoid cross infection of AMF. Tray position on the greenhouse bench was shifted weekly to control for any differences in light or temperature conditions.

The experiment started 52 days after seedling transplantation and lasted for 18 d. Treatment of the plants started in the evening, and was performed as follows: six to seven mycorrhizal and the same number of non-mycorrhizal plants were randomly chosen and moved separately to two different trays. Plants in each tray then were divided into three groups that underwent the following treatments: Group 1 – Mechanical wounding (MW): young and old leaves were evenly damaged by punching ten 4 mm holes per plant with a ticket puncher. The same treatment was repeated on the following day, before the volatile collection started. Group 2 – Herbivory (H): six third-instar *S. littoralis* caterpillars per plant were allowed to feed overnight on the foliage. Group 3 – Control (C): control plants received no damage. The foliage of both the treated and the control plants was enclosed within a cellophane bag (205 x 380 mm, Unipack, Germany) to prevent caterpillars from escaping. In total, 32 mycorrhizal and 32 non-mycorrhizal plants underwent the MW treatment, 29 mycorrhizal and 29 non-mycorrhizal plants underwent the H treatment, and 28 mycorrhizal and 28 non-mycorrhizal underwent the C treatment.

The following morning volatile organic compounds were collected from all plants. Cellophane bags and caterpillars were removed before the volatile collection started.

Plant Volatiles Volatile organic compounds emitted by *P. lanceolata* after herbivory, mechanical wounding, or control treatment were collected in a dynamic headspace collection system located in a growth chamber set at 20°C, 55% relative humidity and 85±5 µmol $m^{-2}s^{-1}$ photosynthetically-active radiation.

Approximately 16 h after the start of the treatments (H, MW, and C), each potted plant was placed individually in a 3 l glass desiccator (Schott, Germany). Each desiccator was tightly closed with a glass lid equipped with a valve that allowed air, which was previously purified through a charcoal filter, to enter the enclosure. The incoming air flux was adjusted to 2±0.3 $lmin^{-1}$. After being in contact with the plant, the air exited the glass cylinder through a collection trap (4 mm diam glass tube containing 30 mg Super Q (ARS, Gainesville, FL, USA), positioned in an opening in the lid.

All VOC collections were performed within a six-hour timeframe from around 10 am to 4 pm to minimize variation in volatile emissions due to plant diurnal rhythm. After 4 ½ h VOC collection, the Super Q traps were eluted with 150 µl dichloromethane that contained 1500 ng nonylacetate as an internal standard.

VOCs were identified with a Hewlett-Packard model 6890 gas chromatograph employing the carrier gas He at 1 ml min^{-1}, splitless injection (injection temperature: 220°C, injection volume: 1 µl), a DB-5MS column (30 m×0.25 mm×0.25 µm film, J & W Scientific, Folsom, USA), and a temperature program from 40°C (2 min hold) to 300°C (2 min hold) with a first gradient of 7°C min^{-1} to

155°C and a second gradient of 60°C min^{-1} to 300°C. Coupled to the gas chromatograph was a mass spectrometer (Hewlett-Packard model 5973) with a quadrupole mass selective detector; transfer line temperature, 270°C; ionization potential, 70 eV; and a scan range of m/z 40–350. For quantification, a GC was coupled to a FID detector operating at 250°C, using the same conditions described above.

VOCs were first identified on the GC-MS by reference spectra in the Wiley and National Institute of Standards and Technology libraries and in the literature (Joulain and König 1998) and by comparison of retention times and mass spectra to those of standards in our collection and others kindly supplied by Wilfried A. König, Hamburg (essential oils of *Oreodaphne porosa* and *Aloysia sellowii*). Quantification of the identified compounds was carried out by comparing the peak areas in the FID traces with that of the internal standard, applying a response factor of 1 for the internal standard, 1.11 for (*Z*)-3-hexenyl acetate, 0.75 for (*E*)-β-ocimene and (*E*)-4,8 dimethyl-1,3,7-nonatriene (DMNT), and 0.74 for all the sesquiterpenes (calculated according to the effective carbon number concept (Scanion and Willis 1985)). In addition to the 6 major compounds discussed in the "*Results"* section, other compounds were identified in a subgroup of the 29 plants subjected to herbivory treatment (*N*=number of individuals from which the particular VOC was identified): limonene (*N*=11), α-copaene (*N*=12), β-elemene (*N*=5), α-humulene (*N*=5), α-muurolene (*N*=2), δ-cadinene (*N*=2). As the sum of these terpenoids never exceeded 7% of the total volatiles, they were not included in further analyses.

Plant Performance After VOC collection, the aboveground parts of all plants were cut at ground level, and the number of leaves and fresh weight were recorded for each plant. In order to estimate the amount of leaf area lost due to caterpillar feeding in the herbivore treatment, the leaves from these plants were aligned on a white board together with a reference area of 2.25 cm^2 and photographed with a digital camera. Digital images were analyzed with Adobe Photoshop (Adobe Systems Incorporated, USA). By referring to the amount of pixels in the reference area, actual remaining leaf areas were determined. Leaf area loss due to caterpillar feeding then was reconstructed by using the remaining leaf area as a template. After photographing, leaves of all plants were frozen in liquid nitrogen and freeze dried. Then, the dry weight of each individual was measured.

Iridoid Glycosides Iridoid glycosides were extracted from 25 mg of freeze dried, finely ground leaf material with 1.8 ml methanol. After 6 h extraction, leaf material was centrifuged at 16000 g, and the supernatant was transferred into clean tubes and evaporated to dryness under nitrogen. The pellet was redissolved in 500 µl of a 20% methanol solution and centrifuged at 16000 g (modified from Marak et al. 2002). Separation of iridoid glycosides was achieved on a Hewlett Packard HP 1100 Series HPLC system with autosampler and diode-array detector. The procedure employed a C-18 reversed phase column (Supelcosil LC18, 250×4.6 mm i.d., 5 µm particle size, Supelco) operated at 1 ml min^{-1} and 25°C. Injection volume was 20 µl. Elution was accomplished with a gradient (solvent A: 0,05% trifluoroacetic acid, solvent B: MeCN) of 0–15 % B (15 min), followed by a cleaning cycle (15–100 % B in 0.5 min, 2.5 min hold, 100 to 0 % B in 0.1 min, 5 min hold). Eluting compounds were monitored at 200 nm, and peaks were identified by match of retention time and UV spectrum with those of commercial standards (catalpol, Wako Chemicals; aucubin, Carl Roth, Germany). Additionally, the identity of catalpol and aucubin was confirmed by LC-MS. Concentrations of iridoid glycosides were calculated by using response curves generated with external standards of catalpol and aucubin.

Mycorrhization Rates For the determination of mycorrhization rate, a subset of 30 mycorrhizal and 30 non-mycorrhizal plants was sampled so that an equal number of individuals from each treatment (H, MW, and C) and from each day of volatile collection were included. Immediately after removal of the leaves, roots were rinsed carefully to wash off the soil, fixed in formaldehyde–acetic-acid [FAA: 6.0% formaldehyde, 2.3% glacial acetic acid, 45.8% ethanol and 45.9% H_2O (v v^{-1})], and stored at 4°C. Fixed roots were washed with distilled water, cut in segments of approximately 1.5 cm, and heated at 90°C for 10 min in 10% KOH. Afterwards, roots were rinsed in tap water, acidified to 3.7% HCl for 10 min, and stained for 11 min in a ready-to-use lactophenol blue solution (Fluka, Switzerland) (Phillips and Hayman 1970). The stain in excess was removed in 50% lactic acid. Total mycorrhizal colonization rates (percentage of the examined root segments with mycorrhizal structures) were determined microscopically using the line intersect method (Phillips and Hayman 1970), modified after Schmitz et al. (1991). Stained root segments from one single plant were densely packed on a microscope slide. A minimum of 300 visual fields per slide were observed at 200×magnification.

Plant and Soil Nutrient Analysis Freeze dried, finely ground plant material (300 mg) and the sand-soil mixture in which the plants grew (500 mg) were analyzed for total carbon and nitrogen content with an elemental analyzer (Vario MAX CNS, Elementar, Hanau, Germany).

Statistical Analysis In order to analyze the influence of mycorrhization and treatments (H, MW, and C) on the amount of 1) the individual volatile compounds, 2) the total amount of terpenes, and 3) the amount of GLVs emitted and corrected for the dry weight of the plants at the same time, analysis of covariance (ANCOVA) was used. Type of treatment and presence of mycorrhiza were considered as fixed factors, and dry weight was fitted in the model as a continuous covariate. Data were either cube root (total terpenes and GLV) or log transformed (single volatiles), and quantities equal to zero were excluded from the analysis of single volatiles to match the prerequisites for ANCOVA. Whenever possible, models were simplified by removing non-significant terms (Crawley 2007). Significance of differences between factor levels were tested by Tukey multiple comparisons of means.

As for volatiles, the influence of mycorrhization and treatments was analyzed on catalpol and aucubin levels. Since IGs are constitutively produced compounds known to accumulate during plant development (Barton 2007), time was fitted to the model as a covariate. Time was indicated as the number of days (1 to 18) from the start of the experiment, i.e., from the day of the treatment of the first set of plants. Catalpol quantities were square-root transformed before analysis to fulfil the requirements for ANCOVA.

Significance of differences in aboveground biomass (dry weight), leaf number, herbivory rates, and carbon and nitrogen content between mycorrhizal and non-mycorrhizal plants was tested by Student's *t*-test.

All statistical analyses were performed with software package R (R Development Core Team (2008), R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.).

Results

Plant Volatiles Plantago lanceolata plants emitted a volatile bouquet dominated by the green leaf volatile (GLV), (*Z*)-3-hexenyl acetate, as well as terpenoids of different classes. In order to assess the effect of mycorrhization and different treatments on the volatile emission of *P. lanceolata*, we quantified the six major volatile compounds, which together make up between 70–90% of the total mixture in herbivory treated and mechanically wounded plants. These include the green leaf volatile (*Z*)-3-hexenyl acetate, the monoterpene (*E*)-β-ocimene, the C_{11} homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), and the sesquiterpenes (*E*)-β-caryophyllene, (*E*)-α-bergamotene, and (*E*)-β-farnesene. The volatile organic compound (VOC) emission profile of *P. lanceolata* was significantly different among plants in the different treatments (Fig. 1a, Table 1). Most strikingly, herbivore-damaged plants emitted much higher levels of terpenoids than mechanically-damaged plants or undamaged controls (Fig. 1a, Table 1).

There were significant differences between mycorrhizal and non-mycorrhizal plants. Mycorrhizal plants emitted about 55% less total terpenoids compared to non-mycorrhizal plants in the herbivory treatment (mycorrhiza: $F_{1,171}=5.11$, $P=0.039$; interaction mycorrhiza:treatment: $F_{2,171}=8.14$, $P<0.001$; Fig. 2a). Among the classes of terpenoids, the emission of sesquiterpenes was 63% lower in mycorrhizal compared to non-mycorrhizal plants after herbivory (Table 1), but the emission of monoterpene, (*E*)-β-ocimene, was not affected by this treatment ($F_{1,83}=0.04$, $P=0.845$, Table 2).

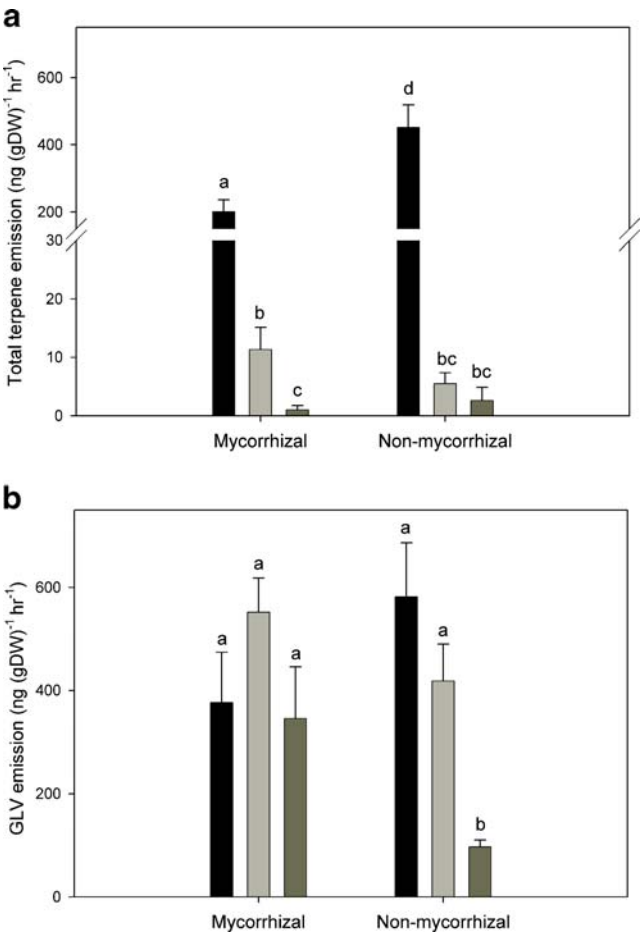

Fig. 1 Total emission of terpenes **(a)** and green leaf volatiles (GLV) **(b)** from *Plantago lanceolata* after herbivory (black bars) and mechanical wounding (light gray bars) in comparison to untreated control plants (dark grey bars). Bars represent means±SE. Different letters indicate significant differences between the means according to ANCOVA followed by Tukey test (adjusted $P<0.05$)

Table 1 Emission rate of the six major volatiles and content of the iridoid glycosides in the leaves of herbivory-treated, mechanically wounded and untreated control *Plantago lanceolata* plants

Compound	Mycorrhizal			Non-mycorrhizal		
	Herbivory	Mechanical wounding	Control	Herbivory	Mechanical wounding	Control
Volatiles						
ng $(gDW)^{-1}hr^{-1}$; mean±SE						
(*Z*)-3-hexenyl acetate	376.61±97.56	552.15±65.96	364.37±99.6	581.96±104.68	376.61±71.3	97.19±13.59
(*E*)-β-ocimene	75.37±15.19	6.94±2.21	1.01±0.71	103.04±17.41	4.06±1.10	0.94±0.60
DMNT	22.05±4.64	0.19±0.19	0.00	68.65±11.71	0.70±0.52	0.00
(*E*)-β-caryophyllene	45.90±10.06	2.23±0.92	0.00	107.69±25.83	0.23±0.19	0.65[a]
(E)-α-bergamotene	25.84±5.03	1.13±0.81	0.00	70.16±12.64	0.45[a]	0.45[a]
(*E*)-β-farnesene	31.84±8.05	0.88±0.61	0.00	101.75±21.67	0.00	0.59[a]
Iridoid glycosides						
µg $(mgDW)^{-1}$; mean±SE						
Catalpol	2.79±0.32	2.87±0.35	3.26±0.34	3.03±0.32	2.95±0.33	2.94±0.47
Aucubin	7.13±0.58	6.61±0.54	6.89±0.49	7.35±0.51	6.54±0.60	5.37±0.54

[a] These volatiles were emitted by a single plant, therefore the SE has not been calculated

For (*Z*)-3-hexenyl acetate, the only major GLV detected, mycorrhizal infection increased emission of undamaged plants more than 3-fold compared to undamaged non-mycorrhizal plants. Herbivory and mechanical wounding of mycorrhizal plants caused no further increase in the emission of this GLV, while these treatments did increase emission from non-mycorrhizal plants by over 5-fold (Fig. 1b).

The ANCOVA showed no significant interactions between the quantity of any of the terpenes and plant dry weight. However, a significant interaction of dry weight and mycorrhization ($F_{4,170}$=4.36, P=0.038, Table 2) was found for (*Z*)-3-hexenyl acetate. This means that the effect of mycorrhization on GLV emission varied according to plant biomass. In small plants, mycorrhization increased (*Z*)-3-hexenyl acetate emission, while it had the opposite effect on larger plants (Fig. 2).

Plant Performance The total aboveground biomass of mycorrhizal plants was on average 8.5% lower than non-mycorrhizal plants (fresh weight: $t_{1,175}$=−3.19, P=0.002, dry weight: $t_{1,176}$=3.87, P<0.001) (Fig 3). This result might be attributed to a drain of fixed carbon toward the fungus in mycorrhizal plants. In fact, mycorrhizal plants contained less carbon in their leaves compared to non-mycorrhizal plants (average C%: mycorrhizal plants= 41.4%, non-mycorrhizal plants=42.3%; Student's *t*-test, $t_{1,24}$=−3.98, P<0.001), but nitrogen content did not vary between leaves of mycorrhizal and non-mycorrhizal plants (N%=0.81% in both groups). The C/N ratio of the sand-soil mixture where the plants were grown was 23.15±0.35 (mean±SE, N=4). Non-mycorrhizal plants had on average a higher number of leaves compared to the mycorrhizal plants (mean non-mycorrhizal: 12.10, mean mycorrhizal: 11.40, $t_{1,162}$=−1.87, P=0.063).

Mycorrhization did not influence the feeding behaviour of *S. littoralis* caterpillars. The amount of leaf tissue consumed overnight was the same in mycorrhizal and non-mycorrhizal plants. ($t_{1,53}$=−0.83, P=0.411).

Iridoid Glycosides The two main iridoid glycosides (IGs) catalpol and aucubin did not show any significant change in

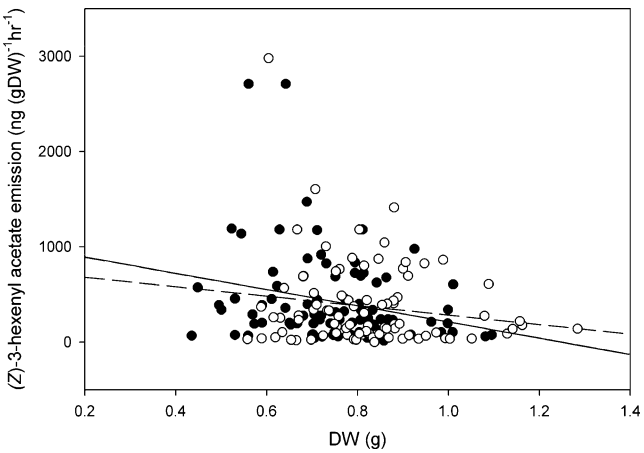

Fig. 2 Linear regression of (*Z*)-3-hexenyl acetate emission and plant dry weight for mycorrhizal (solid line) and non-mycorrhizal (dashed line) plants. Black dots represent all mycorrhizal plants (herbivory-treated, mechanically wounded and untreated), while open dots represent all non-mycorrhizal plants (herbivory-treated, mechanically wounded and untreated)

Table 2 ANCOVA summary table for the effects of mycorrhization, treatment and dry weight on the VOC emission of *Plantago lanceolata*

VOC	Factors				Covariate		df residuals
	Mycorrhiza (df=1)		Treatment (df=2)		Dry weight (df=1)		
	F	*P* [a]	*F*	*P*	*F*	*P*	
(*Z*)-3-hexenyl acetate	2.45	0.119[b]	15.47	**<0.001**[b]	1.34	0.242[b]	170
(*E*)-β-ocimene	0.04	0.845	46.85	**<0.001**	6.78	**0.011**	82
DMNT	7.68	**0.008**	8.55	**0.005**	1.20	0.279	48
(*E*)-β-caryophyllene	11.20	**0.001**	12.98	**<0.001**	3.00	0.089	50
(E)-α-bergamotene	18.14	**<0.001**	1.74	0.185	1.84	0.180	54
(*E*)-β-farnesene	7.57	**0.008**	1.066	0.353	1.19	0.281	46

[a] Bold numbers indicate significant effects

[b] For (*Z*)-3-hexenyl acetate two interactions were significant: mycorrhiza x treatment (F=8.41, P<0.001) and mycorrhiza x dry weight (F=4.36, P= 0.038). There was no significant interaction for any of the other volatile organic compounds

quantity, either between the treatments or between mycorrhizal and non-mycorrhizal plants (catalpol: treatment: $F_{2,173}$=0.065, P=0.937, mycorrhiza: $F_{1,173}$=0.021, P= 0.884; aucubin: treatment: $F_{2,173}$=2.09, P=0.127, mycorrhiza: $F_{1,173}$=0.98, P=0.322) (Table 1). The cofactor 'time' was significant for both catalpol ($F_{1,173}$=11.91, P<0.001) and aucubin ($F_{1,173}$=8.14, P=0.005), indicating that the concentration of IGs in the leaves slightly increased over time.

Mycorrhization Rates At the moment of harvest, all inoculated plants were infected by AMF. On average, we found 68% of the root system colonized by the fungus. No mycorrhizal structures were detected in roots of non-mycorrhizal plants.

Discussion

Mycorrhizal symbiosis is known to affect many plant parameters, including chemical defense (Gange et al. 2007; Hartley and Gange 2009). To understand fully the effects of this symbiosis on chemical defense, it is necessary to investigate compounds involved in both direct and indirect defense. Indirect defense is often manifested by the herbivore-induced release of volatile organic compounds (VOCs) that attract herbivore enemies. The results from our study show that *P. lanceolata* produces a large increase in volatile terpenoids after herbivory that may serve in indirect defense. However, the association of *P. lanceolata* with the arbuscular mycorrhizal fungus (AMF) *G. intraradices* significantly modifies the release of herbivore-induced VOCs from the plant by reducing the emission of the three major sesquiterpenes and the C_{11} homoterpene, DMNT. As DMNT and sesquiterpenes are prominent components of volatile blends known to act in indirect defense (Turlings et al. 1990), AMF may decrease the ability of herbivore-damaged plants to attract herbivore enemies. Consistent with these results, Jallow and co-workers (2008) recently reported that the inoculation of tomato plant roots with the endophytic fungus *Acremonium strictum* lead to a reduction in total terpenoid emission. In *Artemisia annua*, Rapparini et al. (2008) reported that infection with different *Glomus* species constitutively reduced the amounts of sesquiterpenes produced by undamaged plants compared to non-infected control plants. In contrast, infection had no effect on the amount of monoterpenes emitted by *A. annua*. These findings are consistent with our results from caterpillar-infested *P. lanceolata* plants where the major emitted monoterpene ((*E*)-β-ocimene) did not differ in its relative release rate between mycorrhizal and non-mycorrhizal plants.

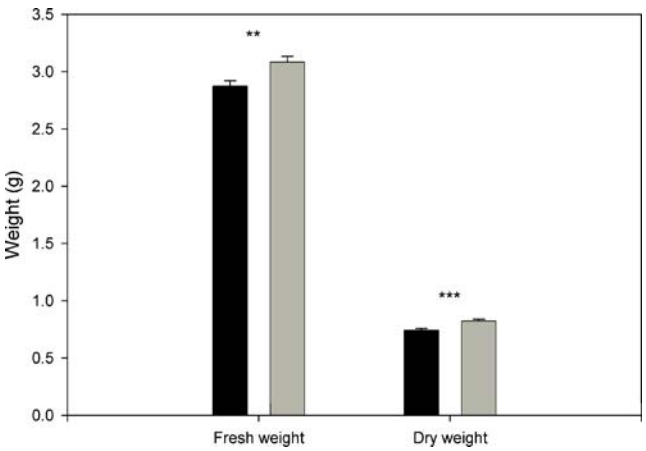

Fig. 3 Aboveground biomass of *Plantago lanceolata*. Fresh weight was recorded once for each plant, on the day the volatile collection was performed. Black bars=mycorrhizal plants, grey bars=non-mycorrhizal plants. Bars represent means±SE. Asterisks represent significant differences according to Student's *t*-test (**P<0.01, ***P<0.001)

Although there is as yet no evidence in the literature that AMF can alter plant indirect defenses, there are reports of the effects of mycorrhization on the major direct defense compounds of *P. lanceolata*, the constitutively produced iridoid glycosides (IGs), aucubin and catalpol. In our study, the levels of IGs were unaffected by mycorrhization. In contrast, a positive effect of mycorrhization on the IG content of *P. lanceolata* was reported by Gange and West (1994), who recorded higher levels of IGs in mycorrhizal compared to non-mycorrhizal plants. The authors attributed this result to the relatively higher fixed carbon levels in the shoots of mycorrhizal plants compared to non-mycorrhizal plants, which allowed more substrate to be allocated to IG production. In contrast, no effect of mycorrhization by *G. intraradices* was found on the catalpol content of *P. lanceolata* leaves by Wurst et al. (2004). Moreover, these authors observed a decrease in leaf carbon content after mycorrhization. We also observed a decrease in leaf carbon levels in the present study, and saw no increase in IG content. This finding supports the hypothesis that IGs are produced in higher amounts after mycorrhization only when symbiosis leads to greater amounts of fixed carbon in the leaves.

Not only did we find a reduction of total leaf carbon content in mycorrhizal plants, but also the aboveground biomass of mycorrhizal plants was lower than that of non-mycorrhizal plants. Although positive effects of AMF on their symbionts' productivity seem to be commonplace (Johnson et al. 1997), neutral or negative effects of mycorrhization on plant growth parameters and biomass also have been documented for *P. lanceolata* (Ayer et al. 1992; Klironomos 2003; Reynolds et al. 2005) and a number of other plant species (see Johnson et al. 1997 and references therein). That AMF can function as carbon sinks in plants has already been documented (Wurst et al. 2004; Reynolds et al. 2005; Ayres et al. 2006). If the growth of the fungus limits the carbon available to the plant for its basic metabolic requirements, the association could turn from symbiosis into parasitism, a phenomenon that has been attributed by some authors to nutrient-rich substrates, low temperatures, or limiting light conditions (reviewed by Smith and Smith 1996, Purin and Rillig 2008) or to particularly high mycorrhization rates (Gange et al. 1999 and references therein). In addition, AMF can be detrimental in association with particular hosts (Klironomos 2003), and the predisposition of a given fungal strain to parasitism rather than symbiosis seems to be at least partially under genetic control (Johnson et al. 1997). When confronted with a non-beneficial AMF association, plants might reallocate their resources among growth, defense, reproduction, and other functions. In this study of young *P. lanceolata*, there was a decline in growth, and a reduction in indirect defenses (volatile terpenes), but no change in the level of direct defenses (iridoid glycosides). These findings support our hypothesis that a net decrease in carbon availability caused by the fungus can cause a reduction in allocation to defense (in this case indirect defense). Maintaining high levels of direct chemical defenses in early developmental stages is a strategy adopted by a number of plant species, for example *Arabidopsis thaliana* (L.) Heynh. (Brown et al. 2003) and *Nicotiana sylvestris* Spegazzini and Comes (Ohnmeiss and Baldwin 2000). The reduction in indirect defenses observed can also be interpreted as a consequence of the general repression of defenses due to AMF colonization. In the first stages of the association, AMF seem to be able to modulate the defense signaling cascades in the plant, and lower the production of defense compounds (reviewed by Garcìa-Garrido and Ocampo 2002).

The mechanisms that underlie the induction of volatile terpene emission in *P. lanceolata* after biotic stresses such as herbivore feeding have not yet been elucidated. Terpene biosynthesis is carried out by condensation of C_5 isoprenoid units, which, in plants, can derive from two pathways: the methylerythritol phosphate (MEP) pathway, considered the principal route to monoterpene production, and the mevalonate (MVA) pathway, thought to be responsible for the production of sesqui- and triterpenes. The MEP pathway also leads to the production of carotenoids and eventually apocarotenoids, some of which are responsible for the yellow-colored roots seen in a number of species after mycorrhization (Strack et al. 2003). Interest in the function of this yellow pigment has stimulated study of the MEP pathway during mycorrhizal formation. Mycorrhization enhances the transcription of genes encoding 1-deoxy-D-xylulose 5-phosphate synthase (DXS), the enzyme that catalyzes the initial step of the MEP pathway in several species including *Medicago truncatula* L., *Nicotiana tabacum* L., *Zea mays* L., *Lycopersicon esculentum* Mill., and *Triticum aestivum* L. (Walter et al. 2000, 2002). An accumulation of transcripts for 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), the enzyme immediately downstream from DXS in the MEP pathway, also has been reported in wheat roots after *G. intraradices* infection (Walter et al. 2000). This AMF-mediated activation of the MEP pathway has not yet been examined with respect to the formation of monoterpenes, assumed to be MEP pathway-derived. After mycorrhizal infection of *P. lanceolata*, we observed no change in volatile monoterpene emission or the accumulation of the IGs, which derive from the same biosynthetic route as monoterpenes. Instead, a decrease in sesquiterpenes and DMNT, assumed to be derived from the MVA pathway, was detected. There are no reports in the literature on how the mevalonate pathway is altered by AMF infection.

The production of IGs is known to be strongly dependent on plant age in *P. lanceolata* (Fuchs and Bowers

2004; Barton 2007). In the study by Gange and West (1994), who found higher levels of IGs in mycorrhizal compared to non-mycorrhizal plants, IGs were extracted from four-month-old plants, which had been associated with AMF for 14 weeks. In contrast, the plants in the study by Wurst et al. (2004), where the IG content did not change with mycorrhization, and in our study were harvested between 9 and 12 weeks of age with a mycorrhizal period of 7 or 10 weeks, respectively. According to Fuchs and Bowers (2004), the concentration of IGs in *P. lanceolata* starts increasing 9 weeks after germination, while Barton (2007) found a strong increase in constitutive levels of IGs already after 6.5 weeks, and furthermore detected a positive correlation between growth rate and daily IG production. Direct comparisons of our results with the literature are difficult because of differences in growing conditions and the genetic properties of the plant material. Nevertheless, it seems plausible that the lack of increase in IGs on mycorrhization observed in our plants is due to harvest at a relatively early developmental stage.

The production of the GLV (*Z*)-3-hexenyl acetate in undamaged mycorrhizal-infected plants was comparable to that in herbivore or mechanically damaged individuals whether or not they were infected with mycorrhizae (Fig 2). Like many volatile terpenes, GLVs have been indicated in attracting insect parasitoids (Hoballah and Turlings 2005; Shiojiri et al. 2006), and can, therefore, be considered indirect defense signals. By increasing the emission of levels of GLVs in undamaged plants, mycorrhizal infection turns these typically induced volatiles into constitutive ones. This constitutively high GLV emission could be a possible explanation for the enhanced attractiveness of mycorrhizal vs. non-mycorrhizal plants to herbivore parasitoids observed by Guerrieri et al. (2004).

Considering the broad distribution and the importance of AMF associations in terrestrial ecosystems in general (Treseder and Cross 2006; van der Heijden et al. 2008) and more specifically in agricultural systems (Sawers et al. 2008), there is a need for further studies to analyze the impacts of mycorrhizal associations on plant defense in a broad range of species and the regulatory mechanisms responsible for these changes. Based on the present results, such investigations should also include an assessment of the mycorrhizal effects on the attraction of herbivore enemies. Since this attraction can vary when a combination of fungal species are associated with one plant (Gange et al. 2003), the effect of simultaneous association of different AMF on plant chemical defenses should also be investigated.

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Chemotaxis Disruption in *Pratylenchus Scribneri* by Tall Fescue Root Extracts and Alkaloids

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Abstract Tall fescue (*Festuca arundinacea*) forms a symbiotic relationship with the clavicipitalean fungal endophyte *Neotyphodium coenophialum*. Endophyte-infected grass is tolerant to nematode, but the factors responsible are unknown. One objective of this work was to determine if root extracts of tall fescue effected chemoreceptor activity of *Pratylenchus scribneri* by using an *in vitro* chemoreception bioassay. Another objective was to determine if specific ergot alkaloids (ergovaline, ergotamine, a-ergocryptine, ergonovine), and loline alkaloids, all produced by the fungal endophyte, altered chemotaxis with this bioassay. Methanolic extract from roots altered chemotaxis activities in this nematode but only from roots of plants cultured 45 ≥ d, which repelled nematodes. Extracts prepared from noninfected grasses were attractants. This assay indicated that the alkaloids were either repellents or attractants. N-formylloline was an attractant at concentrations of 20 μg/ml and lower, while at higher concentrations it was a repellent. Ergovaline, the major ergot alkaloid produced by the endophyte, was repellent at both high and low concentrations and caused complete death of the nematodes.

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Introduction

Tall fescue (*Festuca arundinacea,* synonyms *Lolium arundinaceum* = *Schedonorus arundinaceus*) is a major perennial, cool-season turf and forage grass species that has been established as having a symbiotic relationship with a fungal endophyte *Neotyphodium coenophialum* Glenn, Bacon, & Hanlin (Bacon et al. 1977; Glenn et al. 1996). Subsequent research established that this symbiotum was mutualistic in which the fungus conferred biotic and abiotic defensive metabolites within the relationship (Lyons et al. 1986). The fungal symbiont produces non-conidiating, symptomless intercellular infections of tissue of the leaf sheaths, inflorescence stems, and seeds of tall fescue. The root and leaf blade are not infected. This biotrophic clavicipitalean fungus is the hybrid anamorphic state of several *Epichloe* species that can produce diseases in other grasses and sedges. In tall fescue, the association is entirely symptomless and the fungal symbiont is seed disseminated.

Symbiotic tall fescue is tolerant to numerous abiotic and biotic stresses and diseases, which is attributed to the interactive nature of the endophytic fungus. Of particular importance is that tall fescue is resistant to nematodes (McGlohon et al. 1961). Recent research has suggested that endophyte presence in tall fescue is associated with reduced numbers of various plant-parasitic nematodes in the soil and on grass roots (Bernard et al. 1997). West et al. (1988) and Kimmons et al. (1990) compared population densities of *P. scribneri* in the roots and soil surrounding tall fescue

plants with and without the endophyte. They found fewer nematodes in the endophyte-infected grass roots than in endophyte-free roots, and fewer nematodes were in the surrounding soil of endophyte-infected grass than in endophyte-free grass. This nematode population reduction supports previous findings that endophyte-infected tall fescue may be resistant or tolerant to several species of nematodes (Gwinn et al. 1992). The mechanisms responsible for the above tolerance to nematode infection remain uncertain, but in a recent study (Bacetty et al. 2007; Bacetty 2008), we established that ergot and other alkaloids, as well as tall fescue metabolites, are interactive in the nematode toxicity when measured by an *in vitro* toxicity system.

The lesion nematode, *Pratylenchus scribneri* Steiner, commonly is associated with forage grasses, and has been shown to reproduce on tall fescue (Kimmons et al. 1990; Bernard et al. 1997). *P. scribneri* is an obligate parasite that feeds and migrates in the cortical tissue of plant roots (West et al. 1988; Kimmons et al. 1990; Bernard et al. 1997). They continuously travel within their host's roots, feeding and moving from cell to cell, and can move in and out of the roots traveling freely in the rhizosphere (Bernard et al. 1997). This movement leads to secondary invasion of roots by other soilborne pathogens, which can result in root necrosis. Negative impacts on roots require several mechanisms that are either attractants or deterrents to the feeding activities of this and other nematodes (Roberts et al. 1992).

Since nematode behavior relies on chemoreception to locate potential hosts and other host related interactions (Perry 1996), it is important to know how the endophyte and its associated plant toxins influence nematode behavior. This research was designed to determine the semiochemical nature of specific tall fescue extracts and secondary metabolites from the endophyte to *P. scribneri* in order to provide some insight into the role symbiotic metabolites play in altering chemotaxis of this nematode. This was undertaken with the use of an *in vitro* bioassay that measures the behavior of *P. scribneri*. The specific objectives included: 1) assessing the attraction or repellency of total root exudates from symbiotic and nonsymbiotic tall fescue to *P. scribneri,* and 2) assessing the attraction or repellency to purified alkaloids known to accumulate in symbiotic tall fescue.

Methods and Materials

Nematode Culture Sterile cultures of *P. scribneri* were generously provided by Dr. Susan Meyer (USDA, ARS, Beltsville, MD). These nematodes were cultured on sterile corn root explants following the procedure of Meyer et al. (1990). Corn (*Zea mays* L.) cv. Pioneer 3223 seed were surface disinfected for 10 min in 100% commercial bleach (5.25% sodium hypochlorite) followed by a 15 min soak in 95% ethanol. Nine to ten surface sterilized seeds were placed onto 1.5% water agar (15 g/L, Difco, Detroit, MI, USA) plates and allowed to incubate in the dark at 25°C for 3–5 days. Primary roots were cut and transferred to pH adjusted (5.7) Gamborg's B5 agar (15 g/L) (Gibco, Carlsbad, CA, USA). Plugs from the original Meyer plate were transferred onto the fresh sterile roots. Prior to experiments, *P. scribneri* were extracted from the culture plates by pipetting sterile, distilled water (ca. 25°C) onto the surface of the B5 medium and transferring the nematode solution to sterile conical tubes for a stock solution. Nematodes were diluted and counted to desired numbers for experimental use.

Plant Cultures Twenty 2 wk-old tall fescue, cv. Jesup, seedlings (ten infected and ten non-infected with *N. coenophialum*) were transplanted to individual 20 cm plastic azalea pots filled with sterilized Fafard Professional Potting Mix. The pots were randomly arranged in the Russell Research Center greenhouse in Athens, Ga. Pots were watered 4 days per week to maintain soil moisture and fertilized with Osmocote's Slow Release Plant Food (Marysville, OH, USA) (19-6-12, NPK). Six weeks post transplanting, each pot was repotted into 26 l-plastic pots that would allow for extensive root growth for the entire length of the experiment, usually 10 mo culture. Both plant groups were randomly selected and screened for confirmation of endophyte infection by microscopic examination of the outermost leaf sheath (Hinton and Bacon 1985).

Root Extracts and Alkaloid Preparations Two independent pot culture experiments were conducted to identify the attractive or repellent nature to *Pratylenchus scribneri* from root of *Neotyphodium*-infected and non-infected plants. Samples were taken on 0 day, 30 days, 45 days, and 60 days for total root extract analysis. Total root extracts were prepared from 1.29 g and 9.61 g of roots that were ground in 500 ml of methanol by using a Polytron tissue grinder, filtered (Whatman #2), and the methanol was evaporated to dryness under a vacuum by using a rotoevaporator at 45°C. The residues were weighed, and a stock solution was prepared by dissolving the residue in sterile distilled water and filter sterilized (Acrodisc Syringe Filter 0.2 μm HT Tuffryn Membrane). Stock solutions of total extracts were adjusted with sterile distilled water to yield final test concentrations of 400 μg/ml, 200 μg/ml, 100 μg/ml, 50 μg/ml, 20 μg/ml, 10 μg/ml, and 5 μg/ml, and their effects on nematode behavior were determined by observing movement tracks on 0.4% water agar. One hundred-μl of root extracts were pipetted into 1 cm wells on opposite sides of a 5 cm Petri dish that contained the 0.4% water agar. Approximately 6 nematodes were placed

in the center of the water agar and allowed to move freely for 2 h while incubated in the dark at 25°C and observations were made with the Nikon microscope-camera setup. All experiments were performed in the dark and repeated at least three times.

All ergot alkaloids were obtained from Sigma Inc., St. Louis, MO, USA, as the free bases, with the exception of ergovaline, which was synthetically prepared as the salt of sodium tartrate (Department of Chemistry, Auburn University). These standards were stored under nitrogen at −20°C until use. The loline alkaloid was prepared from seed of endophyte-infected tall fescue, which was formylated with ethylformate and purified by silica gel chromatography that resulted in 94% pure N-formylloline. All compounds were dissolved in sterile distilled water, and these stocks solutions were filter sterilized and appropriate dilutions made with sterile distilled water as indicated below. All solutions and distilled water used as controlled solution were adjusted to pH5.5, which is the resulting pH of extracts obtained from roots during the grinding process. All stock and working solutions of ergot alkaloids were stored at −20°C until use, and these solutions were stable during the 2–4 h observation periods.

Nematode Chemotaxis Assay The effects on nematode behavior were compared by observing movement tracks in thin agar plates, following the procedure of Wuyts et al. (2006). Ergot and loline alkaloids, and extracts prepared as described above from roots of the *Neotyphodium*- infected and non-infected tall fescue cv. Jesup were used in this procedure. To determine at what concentration nematodes were attracted to or repelled by a test compound, 100 μl of the sterile ergot alkaloid solutions including ergovaline, ergotamine, ergonovine, α-ergocryptine, and loline were individually tested at concentrations of 200, 100, 50, 20, 10, 5, and 1 μg/ml. Controls consisted of sterile distilled water, and tartaric acid. A positive control consisted of concentrations of $CaCl_2$.

Observations were made with a Nikon SMZ1500 microscope with a Nikon DXM1200C digital camera attachment. Photographs were downloaded to PowerPoint, and the tracts were traced for ease in presentation. Compounds were determined to be an attractant or a repellent by using the definition and the sixteen-segment grid developed by Wuyts et al. (2006). The grid was placed on an agar plate to record the presence = 1, or absence = 2 of nematode tracks. The technique used to calculate the chemotaxis factor (Cf) was modified to include dividing the sum of scores of the attractant zones by those of the repellent zones. A Cf greater than 2 denoted an attraction, while values lower than 0.5 resulted in the compound being a nematode repellent.

Statistical Analysis Bioassay data were analyzed with regression analyses (SAS v. 9.1, SAS Institute, Carry, NC, USA) to determine significant differences between root extracts and fractions of endophyte-infected or non-infected fescue and their effects on *P. scribneri* motility. Regression testing (SAS v. 9.1) was used to determine significant differences of data generated from *in vitro* bioassays that tested the effects of alkaloids, on *P. scribneri* motility.

Results and Discussion

To reveal more about the biology of nematode suppression, an *in vitro* bioassay was used to evaluate the secondary metabolites within the roots of *N. coenophialum*-infected tall fescue on nematode behavior. From the results (Fig. 1), we determined that nematode behavior is affected by the secondary metabolites produced in this fungal-grass association, and that these factors are significant compared to

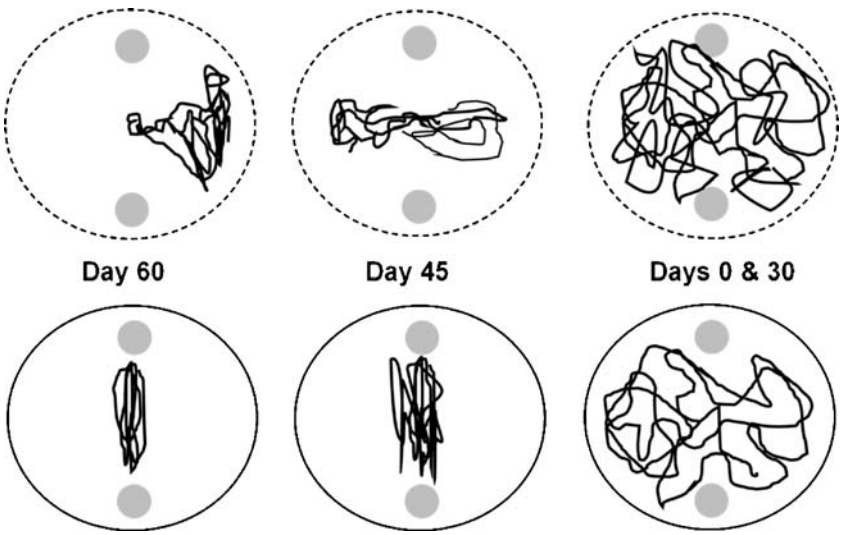

Fig. 1 Chemotaxis in *Pratylenchus scribneri* exposed to a methanolic extract of tall fescue root harvested from endophyte-infected (*dash circles*) and non-infected (*solid circles*) plants cultured over a 60-day growth period. Observations were made during a 2-h observation period

controls ($P<0.05$). After calculating the chemotaxis factors (Cf), extracts from days 0 and 30 exhibited a neutral effect on *P. scribneri* behavior in both the endophyte-infected and non-infected samples. Clear differences between endophyte status treatments occurred with extracts from the 45 day and 60 day sampling periods. Methanol extract of the endophyte-infected roots of 45 day-old tall fescue exhibited strong repellency at 100–400 μg/ml concentrations with Cf scores of 0, while the lower 5–50 μg/ml concentrations were weaker repellents with Cf scores of 0.3. In contrast, non-infected root extracts were strong attractants across all concentrations tested (Cf=3). Day 60, extracts exhibited similar strong nematode attraction across all concentrations (Cf=3). However, in the endophyte-infected root extracts, concentrations 50–400 μg/ml were all strong repellents (Cf=0), while 5–20 μg/ml remained weak repellents (Cf=0.3). Thus, a chemoreceptor substance is temporally expressed in the roots of infected tall fescue. Controls performed as expected, $CaCl_2$, a positive control, was an attractant (Cf=3); sterile distilled water had no effect on behavior as evidenced by nematodes' movement over the entire plate, and tartaric acid at the concentrations used was negative but at concentrations of 0.25% and greater was a strong repellent (Cf=0).

Using the same chemotaxis bioassay system, nematode behavior was examined when exposed to purified alkaloids that are produced in *N. coenophialum*-infected tall fescue. Representative chemotaxis responses to some concentrations are presented in Fig. 2. All produced significant effects ($P<0.05$) compared to comparable controls. Ergovaline, the principal ergopeptide alkaloid of endophyte-infected fescue, is a strong repellent (Cf=0) at the 100–200 μg/ml concentrations and a weak (Cf=0.3) repellent from 1–50 μg/ml. Following the 2-h incubation period, *P. scribneri* at the 10–200 μg/ml ergovaline treatments were found clustered together dead inside the agar. However, we determined that at the 1–5 μg/ml concentrations ergovaline was toxic (Bacetty et al. 2007). Since ergotamine and α-ergocryptine are also ergopeptides, it was expected that they would act as nematode repellents. Across all concentrations, ergotamine was an attractant (Cf=2.5–4), and this attractant resulted in nematode mortality at concentrations of 50–200 μg/ml. Alpha-ergocryptine was a weak (Cf=0.2–0.4) nematode repellent between 50–200 μg/ml and an attractant (Cf=3) between 1–20 μg/ml. Ergonovine was an effective but weak (Cf=0.25–0.3) repellent, and nematode mortality was not affected across concentrations.

The pyrrolizidine alkaloids affected *P. scribneri* behavior in a similar fashion to the ergopeptides, ergovaline and α-ergocryptine. The loline alkaloid produced a strong repellent (Cf=0), between 10–200 μg/ml and a weak repellent (Cf=0.3) at 1–5 μg/ml concentrations. As with ergovaline, nematode mortality was adversely affected. In the 20–200 μg/ml treatments, *P. scribneri* were found clustered together and dead after apparently moving a short distance from the inoculation site. Mortality was not affected at the

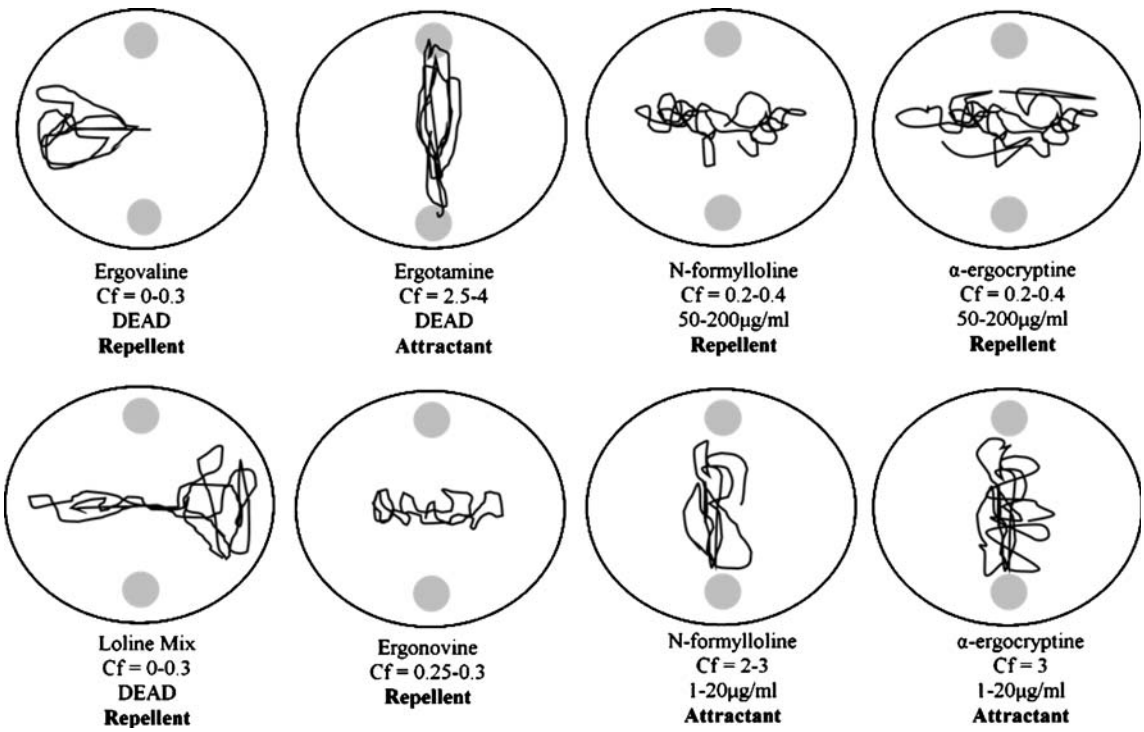

Fig. 2 Selected chemotaxis patterns displayed by *Pratylenchus scribneri* exposed to purified ergot and loline alkaloid concentrations. Observations were made during a 2-h observation period

lower 1–10 μg/ml concentrations. N-formylloline (loline) affected nematode behavior similarly to α-ergocryptine. The higher concentrations, 50–200 μg/ml, were weak (Cf= 0.2–0.4) nematode repellents, while the lower, 1–20 μg/ml, treatments acted as attractants (Cf=2–3). Nematode mortality was not adversely affected across concentrations. All controls (distilled water, pH adjusted distilled water, $CaCl_2$, and 0.01% tartaric acid) had no altering effects on nematode behavior. The data indicate that among the alkaloids the following order of repellency: Ergovaline > Ergonovine > Ergotamine > N-Formyloline > α-Ergocryptine. However, the qualitative and quantitative content of ergot alkaloids contained in leacheates from symbiotic plants within a given pasture situation is dependent on the genetics of the fungal endophyte and its individual grass tiller (Agee and Hill 1994; Hiatt and Hill 1997; Leuchtmann et al. 2000; Salminen and Parwinder 2002, Salminen et al. 2003), which as indicated by these data will produce variations in any disruptive behavior if the desire is for nematode control.

The most biologically active compounds for this nematode are the ergot alkaloids, which are found in abundance in leaves and seed of tall fescue. The natural effects of foliage toxins on a root parasite must be explained if their natural roles in the ecology of root parasitisms are to be understood. *Pratylenchus* species are economically important plant parasites worldwide with a wide host range of monocots and dicots, and depending on species, host, and temperature, the life cycle can be completed in 5–8 weeks. Eggs of this species are laid inside roots, and juveniles go through four molts in a single life cycle. All stages, except the egg, are vermiform, motile, and infective to plant host roots, and as a lesion nematode, migratory endoparasitic behavior results in spending a greater percentage of time within roots, feeding intracellularly within the root cortex. Nematodes are versatile as they can also migrate in and out of the root and sometimes feed as ectoparasites. The result of such destructive behavior alters root anatomy, leading to an unregulated flow of translocates such as ergot alkaloids downward. Further, the feeding behavior of nematodes also serves as a directed sink, pulling above ground substances downward to the roots. Thus, while the ergot alkaloids are produced in the foliage where the endophyte dwells, feeding by this and other species should result in a downward flow of substances, creating root and soil leacheates that contain these and other constituents of symbiotic grasses that are not necessarily translocated downwardly into non-nematode parasitized tall fescue roots. Plant leacheates produced by feeding nematode feeding occur in effective concentrations as they have been attributed to an increase in soil microbial biomass (Denton et al. 1999). Roots of endophyte-infected tall fescue do contain a small amount of ergot alkaloids, although they also contain a significant amount of both N-acetyl and N-formyl loline alkaloids (Bush et al. 1993). However, there are no data on the occurrence and quantities of such compounds in roots of tall fescue parasitized by nematodes species.

The neurotransmitters dopamine, serotonin, and acetylcholine have been detected in several nematode species (Sharp and Akinson 1980; Stewart et al. 2001) where they play a role in several aspects of nematode behavior. In *Caenorhabditis elegans,* the free-living nematode, exposure to exogenous dopamine causes numerous negative effects including paralysis, as well as inhibition of egg laying and defecation (Schafer and Kenyon 1995; Weinshenker et al. 1995). Acetylcholine is involved in locomotion, controlling wave initiation during movement, egg laying, pharyngeal pumping, and in male mating behavior (Hallam et al. 2000; Garcia et al. 2001). Serotonin also is involved in various nematode behaviors such as locomotion, egg laying, and pharyngeal pumping (Loer and Kenyon 1993; Schafer and Kenyon 1995; Weinshenker et al. 1995). Treatment of *C. elegans* with exogenous serotonin caused paralysis, stimulation of pharyngeal pumping in the absence of food, and stimulation of premature egg laying (Loer and Kenyon 1993; Schafer and Kenyon 1995; Weinshenker et al. 1995).

The ergoline ring system of ergot alkaloids is a structural homologue to the neurotransmitters epinephrine, norepinephrine, dopamine, and serotonin. The structural similarities may explain why some ergot alkaloids act as agonists or antagonists on neurotransmitter receptor sites. Based on structures alone, the biogenic amines, dopamine and serotonin, suggest modes of actions due to the ergots ergoline ring. It has been demonstrated that ergovaline, the major ergot alkaloid produced in endophyte-infected tall fescue, acts on a serotonin receptor (Dyer 1993; Larson et al. 1995). In livestock, ergot alkaloids mimic dopamine and exert an agonistic interaction with the D2 dopamine receptor to inhibit acetylcholine and dopamine release, depress chemosensory activity, inhibit norepinephrine release, and reduce prolactin secretions (Cross et al. 1995). The pyrrolizidines (lolines) alkaloids in infected tall fescue are a class of alkaloids that are of fungal origin, structurally distinct, but functionally similar to those found in vascular plants. The biological activity of the individual lolines has not been tied to livestock toxicosis; however, alone or in combination, they exhibit strong anti-insect activity. While N-formyllolline and N-acetylloline did not express any binding affinity to the D2 dopamine receptor (Larson et al. 1999), there are studies that have demonstrated that the lolines do bind to acetylcholine and/or serotonin receptors (Mattocks 1986).

The bioassay used here determined nematode responses to components of root and a fungal endophyte, which are important in terms of developing control strategies based on

disruption of chemoreception of nematodes, indicating the importance of chemotaxis in regulating the movements of nematode species. We have demonstrated that the *in vitro* migratory behavior of *P. scribneri* is altered in the presence of specific ergot and loline alkaloids, as well as tall fescue root extracts. While the *in planta* situation is unknown, the prior published reports of resistance to nematode and the behavior to root extracts from this work add support that substances from roots are responsible for the observed resistance. Analogs of these semiochemicals may have potential as controls for this and other nematodes. We base this hypothesis on our discussion of the possible mechanisms of action relative to structure-activity relationships of the ergot alkaloids and the perception of these substances by this species, which may serve as the basis for the search for other structures that can serve as allelochemicals for nematode control. The mode of action might be related to receptor sites in nematodes, which were not demonstrated in this work, but are expected to vary according to nematode species. If this is correct, it could account for species specificity observed for the tall fescue populations and the varied reactions of nematode species, thus producing an even greater impact as biocontrol strategies.

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Benthic Herbivores are not Deterred by Brevetoxins Produced by the Red Tide Dinoflagellate *Karenia Brevis*

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Abstract Gulf of Mexico blooms of the dinoflagellate *Karenia brevis* produce neurotoxic cyclic polyethers called brevetoxins. During and after a red tide bloom in southwestern Florida, *K. brevis* cells lyse and release brevetoxins, which then sink to the benthos and coat the surfaces of seagrasses and their epiphytes. We tested the possibility that these brevetoxin-laden foods alter the feeding behavior and fitness of a common benthic herbivore within Floridean seagrass beds, the amphipod *Ampithoe longimana*. We demonstrated that coating foods with *K. brevis* extracts that contain brevetoxins at post-bloom concentrations (1 μg g^{-1} drymass) does not alter the feeding rates of Florida nor North Carolina populations of *A. longimana,* although a slight deterrent effect was found at eight and ten-fold greater concentrations. During a series of feeding choice assays, *A. longimana* tended not to be deterred by foods coated with *K. brevis* extracts nor with the purified brevetoxins PbTx-2 and PbTx-3. Florida juveniles isolated with either extract-coated or control foods for 10 days did not differ in survivorship nor growth. A similar lack of feeding response to brevetoxin-laden foods also was exhibited by two other generalist herbivores of the southeastern United States, the amphipod *A. valida* and the urchin *Arbacia punctulata*. Given that benthic mesograzers constitute a significant portion of the diet for the juvenile stage of many nearshore fishes, we hypothesize that the ability of some mesograzers to feed on and retain brevetoxins in their bodies indicates that mesograzers may represent an important route of vertical transmission of brevetoxins through higher trophic levels within Gulf of Mexico estuaries.

Keywords Herbivory · Harmful algal bloom · Seagrass · Epiphytic algae · Benthic food web

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Introduction

Gulf of Mexico blooms of the planktonic dinoflagellate *Karenia brevis* are among the oldest reported harmful algal blooms known (Landsberg et al. 2005), and some evidence indicates that their blooms have increased in both frequency and severity over the past 50 years (Alcock 2007). In the eastern Gulf of Mexico, *K. brevis* blooms typically originate on the west Florida shelf and are carried into coastal waters by prevailing wind and currents (Steidinger et al. 1998). Coastal blooms often are maintained in nearshore waters and shallow-water estuaries for months before subsiding, and thus, can have extensive impact on pelagic and benthic communities across large spatial scales (Tester and Steidinger 1997). The primary ecological impacts associated with *K. brevis* blooms are profound mortality events involving finfish, birds, and mammals (Van Dolah et al. 2001; Flewelling et al. 2005). The toxic

effects of *K. brevis* are due largely to brevetoxins, lipophilic cyclic polyethers that bind to and activate voltage-dependent sodium channels (VDSC), and thereby disrupt regulation of respiration and circulation (Baden 1989; Baden et al. 2005).

Adverse effects of brevetoxins can occur after either direct inhalation or consumption of brevetoxin-laden prey. Although distinguishing the relative importance of inhalation vs. consumption can be difficult, there is evidence that vertical transmission via consumption plays a primary role (Poli et al. 2000; Tester et al. 2000; Van Dolah et al. 2001; Doucette et al. 2005; Fire et al. 2008). In the pelagos, *K. brevis* cells commonly are consumed by zooplanktivorous herbivores (e.g., Naar et al. 2007), even though *K. brevis* is a relatively poor-quality food (Turner and Tester 1997; Tester et al. 2000; Prince et al. 2006) and alters copepod fitness (Breier and Buskey 2007) and swimming behaviors (Cohen et al. 2007). These zooplankton then are consumed by fishes that can accumulate brevetoxins within their own tissues.

On the benthos, brevetoxins are known to pass through benthic food webs via two trophic pathways (see reviews by Van Dolah et al. 2001; Doucette et al. 2005). Filter-feeding bivalves readily consume *K. brevis* cells and accumulate brevetoxins without increasing their mortality rates, thereby facilitating transmission to bivalve consumers such as omnivorous gastropods, fishes, and crabs. In addition, herbivorous fishes and manatees directly consume seagrasses and epiphytes that are coated with brevetoxins as dying cells sink to the benthos, break open, and release brevetoxins. Brevetoxin loads on these benthic plants can be substantial. For example, after a 2002 bloom in southwestern Florida, brevetoxin loads on seagrasses averaged approximately 1 μg g^{-1} drymass, and reached 3.1 μg g^{-1} drymass (Flewelling et al. 2005). The majority of this brevetoxin (~85%) coated epiphytic algae and persisted for more than ten weeks after *K. brevis* cells disappeared. Brevetoxin accumulation on seagrasses and their epiphytes subsequently has been found to be a widespread and common occurrence in southwest Florida during and after *K. brevis* blooms (Flewelling 2008; L. Flewelling, personal communication).

We propose a third pathway by which brevetoxins may be transmitted through benthic food webs, but that has received relatively little attention. Small benthic herbivores known collectively as mesograzers (e.g., amphipods, isopods, and polychaetes; cf. Brawley 1992) live and feed on brevetoxin-laden seagrass and epiphyte tissue, accumulate the toxins, and subsequently transfer brevetoxins to their own predators. Mesograzers are the primary consumers of seagrass epiphytes in many estuarine systems (Jernakoff et al. 1996; Hughes et al. 2004), tend to live several months during which they could potentially accumulate brevetoxin after a bloom relaxes, and are among the predominant prey items of omnivorous and carnivorous fishes, crabs, and shrimps in seagrass beds proximal to the Gulf of Mexico (e.g., Darnell 1961; Virnstein 1987; Motta et al. 1993; Luczkovich et al. 2002). Importantly, juvenile stages of several fish species depend almost entirely on a mesograzer diet (Stoner 1979), thus increasing the likelihood that mesograzers may serve as an important brevetoxin vector in estuarine food webs.

An untested assumption underlying this hypothesis is that benthic herbivores are willing to consume brevetoxin-laden foods. To address this, we pursued a series of feeding and performance assays with the herbivorous amphipod *Ampithoe longimana*, one of the most common herbivores in seagrass beds of the southeastern United States (Nelson 1980; Brooks and Bell 2001; A. McCarty and E. Sotka, personal observation), and we focused on the following questions: First, do amphipods alter their feeding behavior when offered foods coated with *K. brevis* chemical extracts or purified brevetoxin? Second, is amphipod survivorship or growth altered when isolated with foods coated with brevetoxin-laden extracts or with seawater containing solubilized brevetoxins? Third, do amphipods that are continuously fed brevetoxin-laden tissues for days contain measurable levels of toxin? Finally, do other benthic herbivores (the amphipod *Ampithoe valida* and the purple sea urchin *Arbacia punctulata*) show similar feeding responses toward *K. brevis* extracts similar to *A. longimana* ? Our results generally support the notion that benthic herbivores like *A. longimana* readily consume brevetoxin-laden foods, and indicate that benthic mesograzers could serve as important vectors for brevetoxin transmission through estuarine food webs.

Methods and Materials

Collection and Preparation We collected the amphipod *A. longimana* on the west coast of Florida (Tampa Bay 27° 45′ N, 82° 36′ W), the east coast of Florida (Fort Pierce 27° 28′ N, 80° 17′ W), and Bogue Sound, North Carolina (34° 41′ N, 76° 41′ W). In Tampa Bay, amphipods were collected at Lassing Park within seagrass beds of *Thalassia testudinum* and *Halodule wrightii*. Fort Pierce amphipods were collected on the seaweeds *Caulerpa sertulariodes*, *Padina gymnospora*, and *Hincksia feldmania* or in seagrass beds of *Thalassia testudinum* and *Halodule wrightii* at Stan Blum Boat Launch or Jaycee Park. In Bogue Sound, *A. longimana* and *A. valida* were collected at Radio Island Jetty largely from the seaweed *Sargassum filipendula*. The urchin *Arbacia punctulata* was collected from floating docks within Bogue Sound.

All animals were brought to the Grice Marine Laboratory in Charleston, SC, and kept in plastic tubs that

contained unfiltered seawater from Charleston Harbor at ~20°C with a salinity of 26–30 ppt. The water was aerated constantly, and changed approximately once a week. All amphipods were raised on a varied diet of the seaweeds *Sargassum filipendula*, *Hypnea musciformis, Gracilaria tikvahiae,* and *Ulva* (syn. *Enteromorpha*) *intestinalis* collected from Bogue Sound, NC. All seaweeds were washed in freshwater for at least two 30 s intervals in order to eliminate any *Ampithoe longimana* existing on the plants. Cultures were allowed to breed for one to several generations before the experiments began.

The *Ulva intestinalis* tissues used in feeding assays were collected at Radio Island Jetty. The seaweed was rinsed, cleaned of all other biota, and frozen. The frozen alga then was lyophilized at Hollings Marine Laboratory in Charleston, SC, ground into a fine powder with a Wiley mini-mill, and stored at −20°C until use.

A crude brevetoxin extract was prepared from a *K. brevis* Wilson isolate by using a standard solid-phase SPEC extraction (Twiner et al. 2007). In brief, 200 ml of *K. brevis* culture were applied to a pretreated SPEC C18SAR disc (Varian Chromatography, Lake Forest, CA, USA). After a wash with 60 ml of Milli-Q water and 5 min of drying, brevetoxins were eluted from the discs with 20 ml methanol. A total of four extracts were produced from the same culture. The solution concentrations were quantified by using mass spectrometry (Twiner et al. 2007) and an ELISA antibody protocol (Maucher et al. 2007), which has an estimated detection limit of 100 pg / ml. Approximately 97% of the brevetoxins within these extracts was PbTx-1 and PbTx-2, as identified by liquid chromatography / mass spectrometry (following Wang et al. 2004; Zhihong Wang, unpublished data). Purified PbTx-2 and PbTx-3 were obtained from World Ocean Solutions, Wilmington, NC, USA; PbTx-1 was unavailable.

Feeding Assays Assays were performed with *K. brevis* lipophilic extracts that contained brevetoxins or purified brevetoxins. We never offered live *K. brevis* cells because to our knowledge, the cells rarely, if ever, accumulate on seagrass and its epiphytes. We assessed whether *K. brevis* extracts altered herbivore feeding rates by isolating *Ampithoe longimana* individuals on a single food (i.e., a no-choice assay) that was coated with extracts at known concentrations of crude brevetoxin (0.1, 1, and either 8 or 10 μg g^{-1} drymass, depending on the assay) or with ether only. To prepare the brevetoxin-laden food, the correct volume of brevetoxin in methanol was dried by using nitrogen gas, then dissolved in 20 ml of anhydrous ethyl ether and mixed with 1 g of freeze-dried and powdered *Ulva intestinalis*. Ether was removed under rotary evaporation. A solution of 0.18 g agar in 5 ml distilled water was heated to boiling in a microwave oven, cooled, and mixed with 1 g of brevetoxin-coated *Ulva* powder added to 4 ml water. The 10 ml mixture then was poured over window screen and flattened between two pieces of wax paper. After cooling for 30–60 min, the mesh was cut into grids of 6X10 cells (approximately 2 cm^2) and offered to individual amphipods in plastic cups that contained 50 ml of seawater. Ether-only control foods were prepared by rotary-evaporating 20 ml of anhydrous ethyl ether from 1 g of *Ulva* tissue as above. Twenty amphipods from each population were isolated individually with each treatment or control, and placed in the dark at 25°C for approximately 48 h. Previous work had indicated that feeding rates in the dark were higher relative to daytime feeding rates (Amanda McCarty, unpublished data). The number of squares consumed was recorded for all replicates, and a series of nonparametric Kruskal-Wallis tests were performed by using *R* (http://www.r-project.org/) to assess overall differences in feeding rate between control and brevetoxin-coated foods. *Post-hoc* differences were assessed with a series of nonparametric pairwise Wilcoxsan tests.

We assessed whether feeding preference was altered by the presence of crude brevetoxin extracts by offering amphipods a choice of *Ulva* tissue coated with extracts at known concentrations of brevetoxin or with ether only. Foods were created as described for no-choice assays. One 6×5 grid of the brevetoxin treatment (at either 1 or 8 μg g^{-1} drymass) and one control grid were offered to amphipods placed individually in small plastic cups (N=40). For each replicate, the number of cells cleared of food was recorded twice a day, and a replicate was removed from the assay when either nine cells had been removed from either the treatment or the control, or when the sum total of cells removed was ten or more. For each assay, a paired *t*-test was performed by using *R* to determine whether control and treatment foods were differentially consumed.

The application of heated agar did not appear to change the structure of brevetoxins used to produce the test foods, which is consistent with their description as heat-stable toxins (Baden et al. 2005). When heated agar was applied to several samples of purified PbTx-2 and PbTx-3, LC-mass spectrometry detected only these two compounds and a methanol adduct of PbTx-2 that arises from reaction with methanol. There was no detectable open A ring PbTx- 2, 3, oxidized PbTx -2, or open A ring oxidized PbTx-2 (Zhihong Wang, personal communication).

Performance Assay We also assessed the effects of brevetoxin consumption on the survivorship and growth of Florida *A. longimana*. Twenty females carrying eggs were isolated in Petri dishes and allowed time to release their offspring. The mother was removed after the first recorded appearance of juvenile amphipods. The juvenile amphipods were allowed approximately two weeks to grow on a mixed diet of *Sargassum* and *Ulva* before the performance assay began. A dash of cetyl alcohol powder was placed within

each dish to minimize the risk of juvenile amphipods being trapped in surface tension. After two weeks, the juveniles were isolated into separate Petri dishes and allowed one week to feed on fresh *Ulva* collected in Charleston, SC. The amphipods were then placed separately in plastic cups and raised on freeze-dried *Ulva* coated with or without extracts containing 8 μg/g brevetoxin. Because cetyl alcohol powder may preferentially bind to this lipid (T. Leighfield, personal communication), the powder was not added to these experimental cups. The assay was performed on eight juveniles from the Ft. Pierce population and 50 juveniles from the Tampa Bay population, divided evenly between the control and the treatment (n=29). Low numbers of the Ft. Pierce population is attributable to availability in our cultures. The food was prepared as described above in *Feeding Assays* and food and water were changed on day four and eight. The assay persisted for 10 days and was monitored daily for mortality.

To assess growth, pictures of each living amphipod were taken with a Nikon camera at the start of the assay and again at the end of the assay. In each picture, head height was measured at the end of the assay by using ImageJ (http://rsbweb.nih.gov/ij/) and corrected for degree of image magnification. Treatment effects on head height were assessed via an unpaired t-test. Because no females dropped their eggs in either control or treatment groups, we could not assess female fecundity. At the completion of the assay, 13 amphipods from the brevetoxin and control groups were flash-frozen and preserved at −80°C. To confirm that our feeding assays succeeded in exposing these amphipods to brevetoxins, the concentration of brevetoxin in these amphipods then was determined via ELISA. Because the small size of amphipods precluded ELISA analysis of individuals, we grouped one to five amphipods per replicate vials (N=4 and N=3 for brevetoxin and control groups, respectively).

Because brevetoxins can persist in the water column for weeks after a *K. brevis* bloom terminates, we tested the possibility that *A. longimana* amphipod survivorship would be affected by solubilized purified brevetoxins at one and 10 ng / ml. These concentrations were taken from levels recorded during and after a 2002 *K. brevis* bloom in Tampa Bay (Flewelling et al. 2005). However, brevetoxin concentrations in the water column are notoriously variable. For example, during the middle of a 2006 *K. brevis* bloom in northwestern Florida, Naar et al. (2007) measured brevetoxin concentrations in the water column up to ~80 ng / ml. Tester et al. (2008) similarly recorded vertically and horizontally variable brevetoxin concentrations from non-detect to 72 ng/ml in transects through blooms in southwest Florida. Thus, our survivorship assay mimics bloom conditions, but does not assess amphipod response to maximal concentrations that are sometimes found during exceptional blooms. Individual amphipods were isolated within 50 ml seawater inoculated with methanol (at 0.05% concentration), for a final concentration of 0, 1, and 10 ng/ml solubilized purified brevetoxin. We used 2:1 ratio of purified PbTx-2 to PbTx-3, which approximates ratios measured in senescing blooms (Tester et al. 2008). The sample sizes at the inception of the experiment were 32, 26, and 22 for the 0, 1, and 10 ng / ml treatments, respectively. Mortality was assessed every one to four days for 10 days, and analyzed via a χ^2 test.

Results

In a series of no-choice feeding assays, *Ampithoe longimana* individuals from three locations (St. Petersburg and Fort Pierce, Florida and Morehead City, North Carolina) consumed statistically equivalent amounts of control foods or foods coated with *K. brevis* extracts at brevetoxin concentrations of 0.1 and 1 μg g^{-1} (Fig. 1). Given that the brevetoxins persisted on epiphytes scraped from seagrass blades at concentrations up to 3 μg g^{-1} (Flewelling et al. 2005), these results indicate that observed post-bloom concentrations of brevetoxin do not tend to alter feeding rates of this benthic herbivore. At concentrations several-fold greater than observed post-bloom concentrations (i.e., 8 and 10 μg g^{-1}), there was a tendency for Florida and North Carolina individuals to feed at lower rates on foods coated with *K. brevis* extracts relative to control foods. However, the inhibitory effect of the *K. brevis* extract on Florida populations was not consistent across populations. For example, the St. Petersburg population fed significantly less on foods coated with 10 μg g^{-1} during one assay (Fig. 1a), but showed no tendency for lower feeding rates when foods were coated at 8 μg g^{-1} during an assay performed several months later (Fig. 1b).

When offered a choice between control foods or extract-coated foods with brevetoxin concentrations of 1 μg g^{-1}, all three populations of *A. longimana* amphipods consumed significantly more control foods (Fig. 2). Surprisingly, however, when the assay was repeated two days later at a higher concentration (8 μg g^{-1}), Florida populations fed on control and extract-coated foods at statistically equivalent rates. A likely explanation for inconsistent feeding deterrency in no-choice (Fig. 1) and choice (Fig. 2) assays using *K. brevis* extracts is the presence of unstable deterrent compounds that elute with 100% methanol and that are unrelated to the brevetoxins. We, therefore, tested the effects of purified brevetoxins on *A. longimana* feeding behavior by offering two Florida populations feeding choices between foods coated with PbTx2 or PbTx3 at 1 μg g^{-1} and control foods. Amphipods did not discriminate between control and PbTx2-coated foods, and significantly preferred the PbTx3-coated to control foods (Fig. 3).

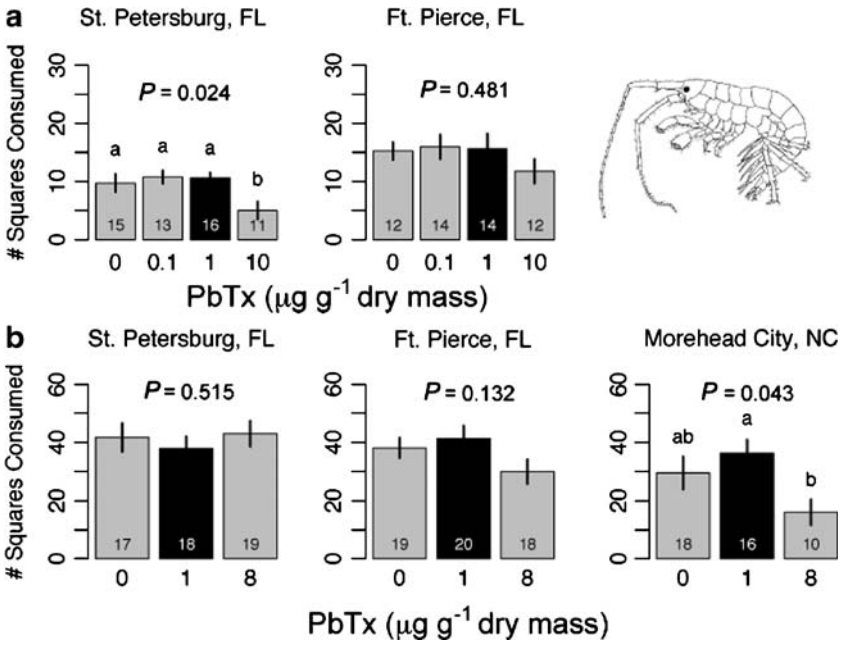

Fig. 1 No-choice feeding assays in which the lipophilic extract of *Karenia brevis* was offered to populations of the herbivorous amphipod *Ampithoe longimana*. Individual amphipods were isolated on freeze-dried *Ulva* (syn. *Enteromorpha*) *intestinalis* tissue coated with ether only ("0"), or with *K. brevis* extracts at a concentration of 1, 8, or 10 μg brevetoxin (g^{-1} dry mass). The *P*-values for Kruskal-Wallis non-parametric tests are indicated, and letters indicate treatments that are statistically indistinguishable by a pairwise Wilcoxan tests. **a**) and **b**) represent assays on independent *K. brevis* extracts and amphipod cultures. The dark bars (1 μg g^{-1}) represent observed concentrations of brevetoxins after bloom events in western Florida

In order to assess whether consuming *K. brevis* extracts has consequences for the survivorship and growth of *A. longimana*, we isolated individual juveniles with foods coated with extracts at 8 μg g^{-1} brevetoxin concentration or control foods. After 10 days, survivorship of Florida *A. longimana* did not differ between treatments (Fig. 4a: χ^2 test: $P>0.100$), nor did size [Mean growth in head height ± S.E. on control foods (0.113±0.021 mm) and extract-coated foods (0.135±0.023 mm); unpaired t-test $P=0.488$)].

To confirm that our feeding assays succeeded in exposing these amphipods to brevetoxins, we froze the juveniles at the end of the performance assay and measured brevetoxin loads within tissues. Juveniles fed brevetoxin-coated foods contained between 2–6 ng g^{-1} wetmass of brevetoxin ($N=4$; data not shown), while juveniles without foods had no detectable brevetoxins ($N=3$). Because individual amphipods averaged 2.8 mg wet mass, each amphipod contained approximately 0.01 ng brevetoxin. Our methods did not distinguish whether these brevetoxins were held in the gut, amphipod tissue, or both.

We also tested whether there was significant mortality when adult amphipods were exposed to seawater with observed concentrations of solubilized brevetoxin (1 or 10 ng ml^{-1}). After 10 days of exposure, control and brevetoxin-laden waters did not alter the survivorship of adult amphipods (Fig. 4b; χ^2 test: $P>0.100$).

To assess whether the lack of a feeding response to brevetoxin-coated foods is common to other generalist benthic

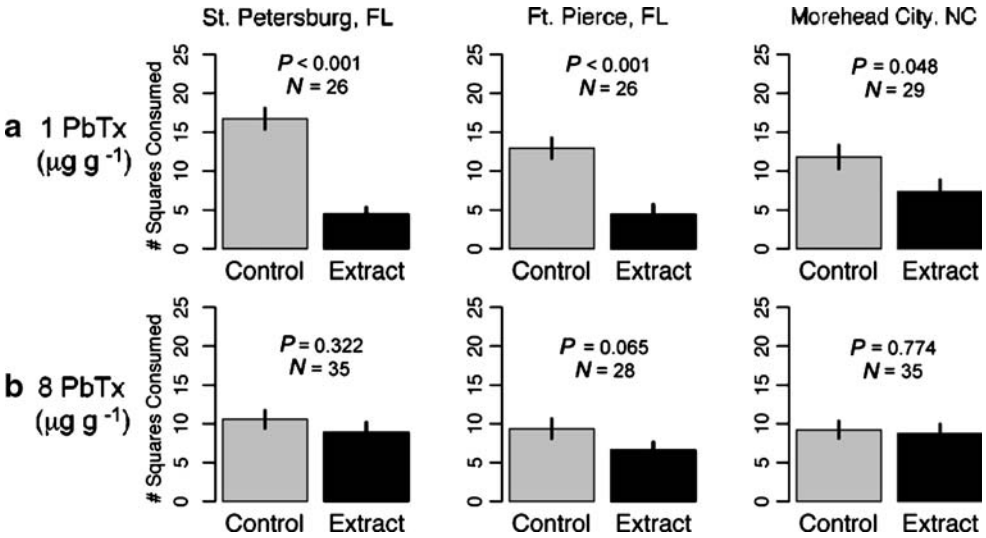

Fig. 2 Pairwise feeding choice by populations of the herbivorous amphipod *Ampithoe longimana*. Individuals were offered a feeding choice between freeze-dried *Ulva* (syn. *Enteromorpha*) *intestinalis* tissue coated with ether only (i.e., Control) or coated with *Karenia brevis* extracts at a concentration of **a**) one or **b**) eight μg brevetoxin (g^{-1} dry mass). Sample sizes and *P*-values from paired *t*-tests are indicated

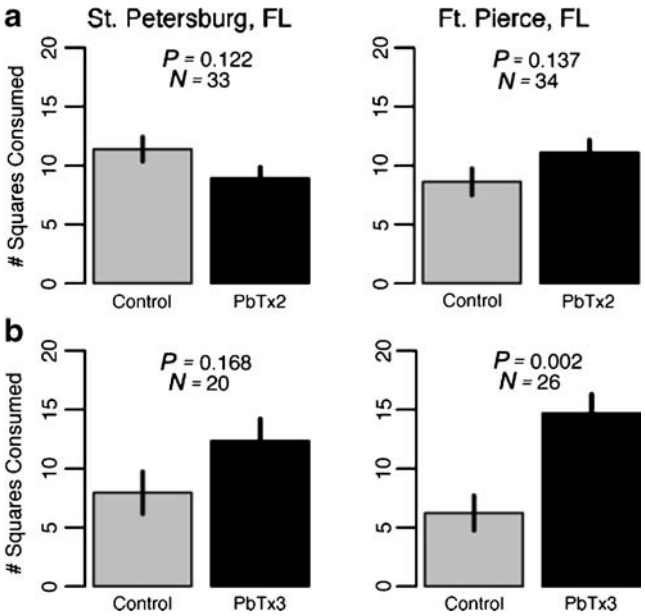

Fig. 3 Pairwise feeding choice by populations of the herbivorous amphipod *Ampithoe longimana*. Individuals were offered a feeding choice between freeze-dried *Ulva* (syn. *Enteromorpha*) *intestinalis* tissue coated with ether only (i.e., Control) or coated with **a**) PbTx2 or **b**) PbTx3 at a concentration of 1 μg brevetoxin (g^{-1} dry mass). Sample sizes and *P*-values from paired *t*-tests are indicated

herbivores, we offered a feeding choice between control and brevetoxin-coated foods at 1 μg g^{-1} to North Carolina populations of the sea urchin *Arbacia punctulata* and amphipod *Ampithoe valida.* Both herbivores readily consumed brevetoxin-coated foods at a rate equivalent to control foods (Fig. 5).

Discussion

As a first step toward understanding the potential importance of mesograzers in the vertical transmission of brevetoxins through benthic food webs of the Gulf of Mexico, we demonstrated that foods coated with observed 'post-bloom' concentrations of brevetoxins do not generally alter the feeding behaviors, survivorship, or growth of the benthic herbivorous amphipod *Ampithoe longimana* (Fig. 1–3, 4a). Moreover, adult amphipods revealed no decline in survivorship when exposed to solubilized brevetoxin at concentrations observed after blooms (Fig. 4b). We also found no detectable effects of *K. brevis* extracts on the feeding choices of two other benthic herbivores, the amphipods *A. valida* and purple sea urchin *Arbacia punctulata* (Fig. 5). Together, these results indicate that after *K. brevis* red tides, benthic herbivores readily feed on brevetoxin-coated foods, survive, and grow in brevetoxin-contaminated waters, and can, therefore, potentially transmit these brevetoxins to their predators.

Although the overall pattern is one of a lack of feeding deterrency, crude *K. brevis* extracts occasionally and inconsistently deterred amphipod feeding (see Results). Several lines of evidence suggest that this inconsistent feeding deterrence is mediated by unstable compounds eluted with 100% methanol extracts that are not brevetoxins. First, we found no change in total brevetoxin concentrations in the crude extracts over eight months of storage and handling (data not shown). Second, a direct test indicated that *A. longimana* was never deterred by purified brevetoxins at observed post-bloom concentrations (Fig. 3). In fact, the presence of PbTx-3 at 1 μg g^{-1} was a feeding attractant to a Fort Pierce population. Finally, we know that our methods were successful in offering brevetoxin-coated foods to the amphipods, because we detected brevetoxins within the tissues and/or guts of amphipods that were isolated on brevetoxin-laden foods for several weeks (see Results). The presence of unstable non-brevetoxin feeding deterrents similarly was inferred by Kubanek et al. (2007) when studying the feeding behaviors of rotifers.

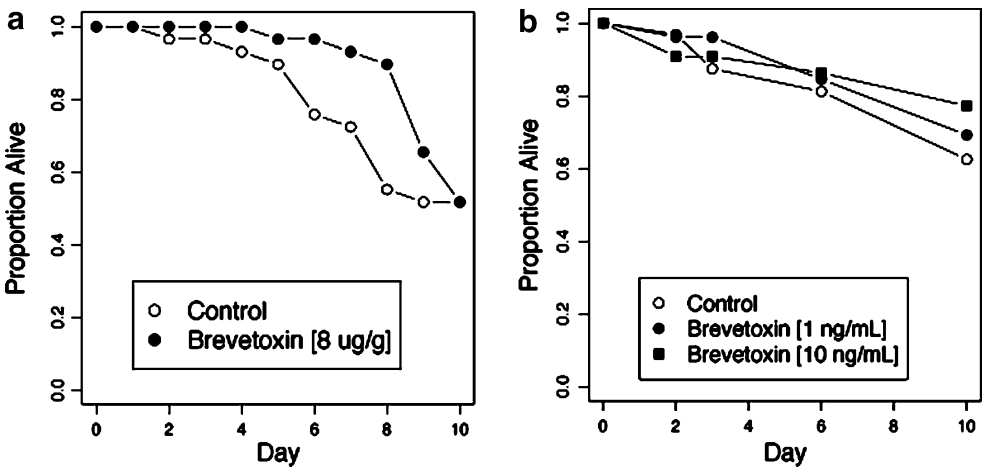

Fig. 4 Survivorship of Florida *Ampithoe longimana* when exposed to brevetoxins. **a**) Survivorship of juvenile amphipods when isolated on *Ulva* (syn. *Enteromorpha*) *intestinalis* tissue coated with ether only (i.e., Control) or *Karenia brevis* extracts at a concentration of eight μg brevetoxin (g^{-1} dry mass). **b**) Survivorship of amphipods when exposed to water with ethanol only (control), or observed (1 or 10 ng ml^{-1}) concentrations of purified brevetoxins

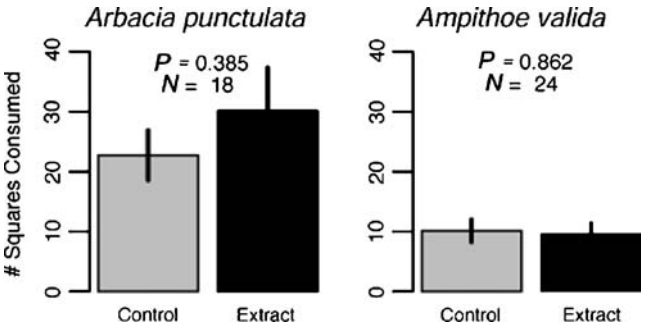

Fig. 5 Pairwise feeding choice by the temperate urchin *Arbacia punctulata* and the amphipod *Ampithoe valida*. Individuals were offered a feeding choice between freeze-dried *Ulva* (syn. *Enteromorpha*) *intestinalis* tissue coated with ether only (i.e., Control) or coated with *Karenia brevis* extracts at a concentration of one μg brevetoxin (g^{-1} dry mass). Sample sizes and *p*-values from paired t-tests are indicated

The lack of deterrent or deleterious effects of brevetoxins is surprising for several reasons. First, these results are inconsistent with observations of elevated mortality among benthic invertebrates when *K. brevis* blooms enter nearshore estuaries (e.g., Simon and Dauer 1972). However, it is difficult to assess whether our data and the mortality events truly are in conflict because brevetoxins loads were not measured during such events, and alternative or compounding explanations also could account for the catastrophic mortality event (e.g., hypoxia: Santos and Simon 1980). Second, the lack of a feeding deterrence seen in *A. longimana* appears to contrast strongly with the strongly deterrent effects of brevetoxins on other marine organisms. *Karenia brevis* cells and extracts appear to alter the behavior and fitness of pelagic rotifers (Kubanek et al. 2007) and copepods (Turner and Tester 1997; Landsberg et al. 2005; Prince et al. 2006; Breier and Buskey 2007; Cohen et al. 2007). In benthic habitats, *K. brevis* cells alter the feeding behavior, abundance and/or fitness of bivalves (Summerson and Peterson 1990; Keppler et al. 2006; Leverone et al. 2006; Haubois et al. 2007; Leverone et al. 2007). The causes of differences in feeding response toward *K. brevis* between groups of herbivores remain unclear, but we find it interesting that the previously studied herbivores consume live *K. brevis* cells, while the benthic herbivores studied here consume brevetoxin-laden epiphytes and seagrasses. Live *K. brevis* may occur occasionally on benthic plants, but unlike *Gambierdiscus* on tropical macroalgae (Cruz-Rivera and Villareal 2006), *K. brevis* does not sustain benthic populations.

We were also surprised that we could not detect significant differences in feeding behavior among amphipod populations that are annually exposed to *K. brevis* blooms (i.e., Tampa Bay; (Brand and Compton 2007) versus populations that rarely experience such blooms [i.e., Fort Pierce, Florida and Morehead City, NC; (Summerson and Peterson 1990)]. We had expected such microevolution to occur, because this amphipod has evolved population-level differences in feeding behaviors and fitness when isolated with lipophilic secondary metabolites produced by the brown seaweed *Dictyota* (Sotka and Hay 2002; Sotka et al. 2003; Sotka and Whalen 2008), and because some populations of a benthic clam (*Mya arenaria*; Bricelj et al. 2005) and pelagic copepod (*Acartia hudsonica*; Colin and Dam 2002) have evolved tolerance toward harmful algal blooms in the genus *Alexandrium*. It remains possible that population-level variation in *A. longimana* fitness traits is present (e.g., female fecundity) but this was not tested here.

A review by Landsberg (2002) concluded that the overall effects of harmful algal blooms (or HABs) "on food webs and ecosystems are probably the least understood of all impacts," and that "the pathways of algal-toxin transmission (are) complex and incompletely known" (pg. 188; see also Hay and Kubanek 2002; Cruz-Rivera and Villareal 2006). We propose that mesograzers and other benthic herbivores may represent an underexplored set of pathways by which brevetoxins produced by Gulf of Mexico *K. brevis* red tide blooms are transmitted vertically through estuarine food webs. Central to our hypothesis is the fact that mesograzers are a vitally important component of the diet for a variety of nearshore fishes (Motta et al. 1993; Luczkovich et al. 2002; Marancik and Hare 2007), including the extremely abundant pinfish *Lagodon rhomboides* (Stoner 1979). Moreover, the importance of mesograzers as a diet is greater for juvenile than adult life stages, suggesting that younger fish will be particularly vulnerable to brevetoxin transmission via mesograzer consumption. In support of our hypothesis, Naar et al. (2007) and Fire et al. (2008) document substantial loads of brevetoxins within *L. rhomboides* and other fishes in western Florida estuaries when collected weeks after a *K. brevis* blooms had relaxed.

We recognize that acceptance of benthic mesograzers as an additional mode of brevetoxin transmission requires answers to several outstanding questions. We do not know whether natural populations of mesograzers are laden with brevetoxins. We also do not know the rates at which bioaccumulation (if present) and depuration occurs in mesograzers. The brevetoxin load we report is much lower than that detected for pelagic copepods under laboratory conditions (~25 ng brevetoxin per copepod; Tester et al. 2000), although it is not clear whether these experiments (including the present study) utilized ecologically-realistic feeding rates. Moreover, we have not demonstrated that consumption of brevetoxin-laden mesograzers by fishes actually yields accumulation in fishes (e.g., Tester et al. 2000; Naar et al. 2007). Nevertheless, the present study does indicate that mesograzers deserve attention in our efforts to predict bioaccumulation rates and ecological impacts of brevetoxins within nearshore systems.

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RETRACTION NOTE

Intraspecific and Interspecific Interactions Mediated by a Phytotoxin, (-)-Catechin, Secreted by the Roots of *Centaurea maculosa* (Spotted Knapweed)

Tiffany L. Weir · Harsh Pal Bais · Jorge M. Vivanco

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Erratum to: J Chem Ecol (2003) 29:2397–2412
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This article has been retracted by Tiffany L. Weir and Jorge M. Vivanco.

Reason: After a reevaluation of this work, we no longer have confidence in several key observations and therefore, retract this paper. Specifically, the reported finding in Perry et al. (J of Chemical Ecology 33:2337–2344, 2007) of catechin in experimental blanks, suggest an exogenous source of catechin in our laboratory. The absence of experimental blanks in the current study along with the unreproducibly high levels of catechin reported from *in vitro* grown *C. maculosa* plants have led us to believe that the reported catechin may have originated from non-plant sources. In addition, a finding that some of our commercially purchased catechin was contaminated with 2,4-D along with our observation that catechin caused callus formation in some seedlings suggests that at least some of these experiments may have been conducted using a contaminated source. Because we cannot be sure that contaminated catechin was not used, the results of the phytotoxicity and germination inhibition experiments are meaningless.

The online version of the original article can be found at http://dx.doi.org/10.1023/A:1026313031091.

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Alkyldimethylpyrazines in the Defensive Spray of *Phyllium westwoodii*: A First for Order Phasmatodea

Aaron T. Dossey · Marco Gottardo · John M. Whitaker · William R. Roush · Arthur S. Edison

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Abstract *Phyllium westwoodii* is a phasmid insect (Order Phasmatodea) belonging to the Family Phylliidae (leaf insects). These rather large and ornate creatures are known for their morphological resemblance to plant leaves for camouflage. Pyrazines are a common class of compounds used or produced by a wide variety of organisms, even humans. When an individual of *P. westwoodii* is disturbed, it sprays an opaque liquid from a pair of prothoracic glands, which are utilized by other phasmid species for defense. The current study has found that this liquid contains glucose and a mixture of 3-isobutyl-2,5-dimethylpyrazine, 2,5-dimethyl-3-(2-methylbutyl)pyrazine, and 2,5-dimethyl-3-(3-methylbutyl)pyrazine. This is the first report of pyrazines found in the defensive gland spray of phasmid insects, and the first chemical analysis of glandular material from family Phylliidae.

Keywords Insect · *Phyllium westwoodii* · Phasmatodea · Phasmid · Phylliidae · Chemical defense · Dimethyl alkylpyrazine · 3-isobutyl-2,5-dimethylpyrazine · 2,5-dimethyl-3-(2-methylbutyl)pyrazine · 2,5-dimethyl-3-(3-methylbutyl)pyrazine · Glucose

Introduction

Chemical defense in walkingstick insects (also called stick insects, or "phasmids"; Order Phasmatodea) has been studied for decades. However, the chemical composition of defensive sprays from only a few species has been analyzed (Schneider 1934; Meinwald et al. 1962; Smith et al. 1979; Chow and Lin 1986; Ho and Chow 1993; Bouchard et al. 1997; Eisner et al. 1997; Dossey et al. 2006, 2007; Schmeda-Hirschmann 2006). The first analysis of a phasmid defensive spray was published in 1934 on *Agathemera crassa* (Schneider 1934) (referred to by Schneider as *Paradoxomorpha crassa*). Possibly, due to analytical limitations of that time, the chemical structure was likely incorrect, as the defense compound of another species in that genus (*Agathemera elegans*) was more recently determined to be 4-methyl-1-hepten-3-one (Schmeda-Hirschmann 2006). Subsequent to the work by Schneider in 1934, the next species studied was *Anisomorpha buprestoides* in classic works by Eisner, Meinwald, and co-workers (Meinwald et al. 1962; Eisner 1965). *Anisomorpha buprestoides* produces at least three stereoisomers of a monoterpene first identified by Meinwald et al. (Meinwald et al. 1962; Dossey et al. 2006; Dossey et al.

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2008) and named anisomorphal. Since then, other phasmids have been shown to produce monoterpenes such as: iridodial (*Graeffea crouani*) (Smith et al. 1979), nepetalactone (*G. crouani*) (Smith et al. 1979), actinidine [*Megacrania alpheus* (Chow and Lin 1986) and *Megacrania* tsudai (Ho and Chow 1993)], limonene (*Sipyloidea sipylus*) (Bouchard et al. 1997), parectadial (a novel monoterpene first identified from the phasmid *Parectatosoma mocquerysi*) (Dossey et al. 2007), dolichodial (isomer of anisomorphal found in young *A. buprestoides*) (Dossey et al. 2008), peruphasmal (isomer of anisomorphal from *Peruphasma schultei*) (Dossey et al. 2006, 2008), and others as minor components (Ho and Chow 1993; Bouchard et al. 1997). In addition to monoterpenes, 4-methyl-1-hepten-3-one (as mentioned above) (Schmeda-Hirschmann 2006) and quinoline (from *Oreophoetes peruana*) (Eisner et al. 1997) also have been found in phasmid insect defense gland sprays.

Besides these secondary metabolites, glucose has been reported in the defensive spray of *A. buprestoides* (Dossey et al. 2006; Zhang et al. 2007), *P. schultei* (Dossey et al. 2006), and *P. mocquerysi* (Dossey et al. 2007). The presence of glucose may be of significance in the biosynthesis and/or transport of defensive substances into the glandular reservoir of these insects (see "Discussion") (Dossey et al. 2006, 2007). It also has been shown that *A. buprestoides* has the ability to biosynthesize its defensive monoterpenes from acetate and mevalonate (Meinwald et al. 1966) and *de-novo* from glucose (Dossey et al. 2008). To date, despite the range of small molecules already found in phasmids, no pyrazines have been isolated from insects in the order Phasmatodea.

Leaf insects (Order Phasmatodea, Family Phylliidae) make up a small group of anciently diverged phasmids that comprises 46 known species, all characterized by remarkable cryptic morphological and behavioral adaptations. They have dorso-ventrally flattened bodies and foliaceus lobes on legs that resemble leaves. Females are always much longer and proportionally broader than males, and their antennae also are particularly dimorphic. Adult males have long and densely setose antennae, while adult females possess very short antennae that bear on the third segment a stridulatory apparatus used for the production of defensive sounds (Henry 1922; Bedford 1978; Zompro and Grösser 2003, and references therein). Members of this family now occur in tropical Asia and Australia, with presumably introduced populations in eastern Africa, Madagascar, and adjacent islands, but they once lived as far away as Europe (Wedmann et al. 2007).

Phyllium (Phyllium) westwoodii (Wood-Mason 1875) (Fig. 1A) is a leaf insect that occurs in the Andaman Islands, China, Laos, Myanmar, Thailand, and Vietnam. When threatened, females of *P. westwoodii* enter into an immobile behavioral state known as catalepsy. If the threatening stimulus persists, they begin to stridulate with the antennae, and release an opaque-milky spray from a pair of glands at the anterior of their prothorax (Fig. 1B). This secretion is sprayed at moderate distance over the dorsal surface of the head and thorax. Many other phasmid species produce chemical sprays from homologous glands in response to being disturbed (Schneider 1934; Meinwald et al. 1962; Eisner 1965; Bedford 1978; Smith et al. 1979; Chow and Lin 1986; Ho and Chow 1993; Bouchard et al. 1997; Eisner et al. 1997; Thomas 2001; Dossey et al. 2006, 2007; Schmeda-Hirschmann 2006), and in certain cases these sprays function to repel potential predators (Eisner 1965; Carlberg 1985a b, 1986, 1987; Chow and Lin 1986; Bouchard et al. 1997; Eisner et al. 1997). The odor of *P. westwoodii* defensive spray has a striking similarity to that of chocolate.

Pyrazines are produced by a wide range of organisms (Wheeler and Blum 1973; Brown and Moore 1979; Cross et al. 1979; Blum 1981; Moore et al. 1990; Dickschat et al. 2005a, b). For insects, they often function as pheromones. Alkyldimethyl-pyrazines are used as pheromones by a number of insect species in the Order Hymenoptera (ants, bees, and wasps). Specifically, as trail [leaf cutter ant, *Atta sexdens rubropilosa* (Cross et al. 1979)] and alarm (Wheeler and Blum 1973; Brown and Moore 1979) pheromones by some ant species, and they are found commonly in ant glandular secretions (Blum 1981). Other pyrazines, such as methoxyalkylpyrazines, also protect some insects from predation by functioning as anti-feedants in combination with bright coloration in aposematic species (Moore et al. 1990). These have been found in crude extracts of insects other than phasmids such as grasshoppers [genus *Poekilocerus*, (Moore et al. 1990)] belonging to the Order Orthoptera, a distinct taxon from Order Phasmatodea (Terry and Whiting 2005).

Pyrazines have different uses for humans. They are often associated with characteristic aromas and flavors of some of our favorite foods (Maga and Sizer 1973; Adams et al. 2002). The Flavor and Extract Manufacturer's Association (FEMA) reports that 2,135 kg of pyrazine flavor additives are used in the United States annually (Adams et al. 2002). Alkyldimethylpyrazines function as odorants in foods such as chocolate (Welty et al. 2001) and are produced in the Maillard reaction during the roasting of cocoa beans (Arnoldi et al. 1988). Pyrazines also contribute to the flavor and aroma of roasted peanuts, beef, and other cooked foods (Maga and Sizer 1973).

This study reports the identification of three alkyldimethylpyrazines (Fig. 2), along with glucose, in less than 1 μl of the defensive spray from a single female *P. westwoodii*. Specifically, 3-isobutyl-2,5-dimethylpyrazine (**1**), 2,5-dimethyl-3-(2-methylbutyl)pyrazine (**2**), and 2,5-dimethyl-3-(3-methylbutyl)pyrazine (**3**) (Fig. 3) were

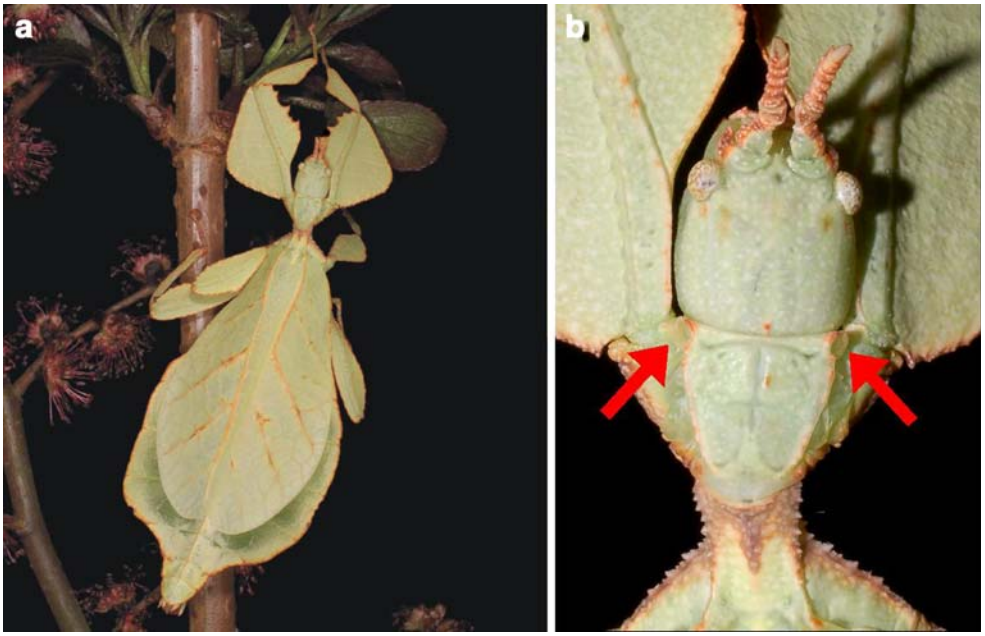


Fig. 1 Photographs of an adult female *Phyllium westwoodii*. **a** Whole insect sitting on *Sambucus nigra* (not native food plant), and **b** close-up of head and prothorax of same insect as in (**a**) with arrows showing the position of the openings of its defensive glands

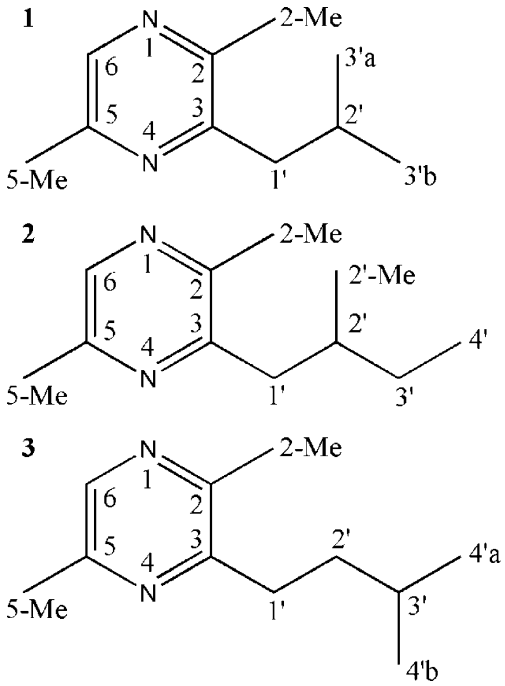


Fig. 2 Three alkyl dimethylpyrazines identified in the defensive spray of *Phyllium westwoodii*. 3-isobutyl-2,5-dimethylpyrazine (**1**), 2,5-dimethyl-3-(2-methylbutyl)pyrazine (**2**), and 2,5-dimethyl-3-(3-methylbutyl)pyrazine (**3**). Each structure is shown with corresponding carbon atom labeling

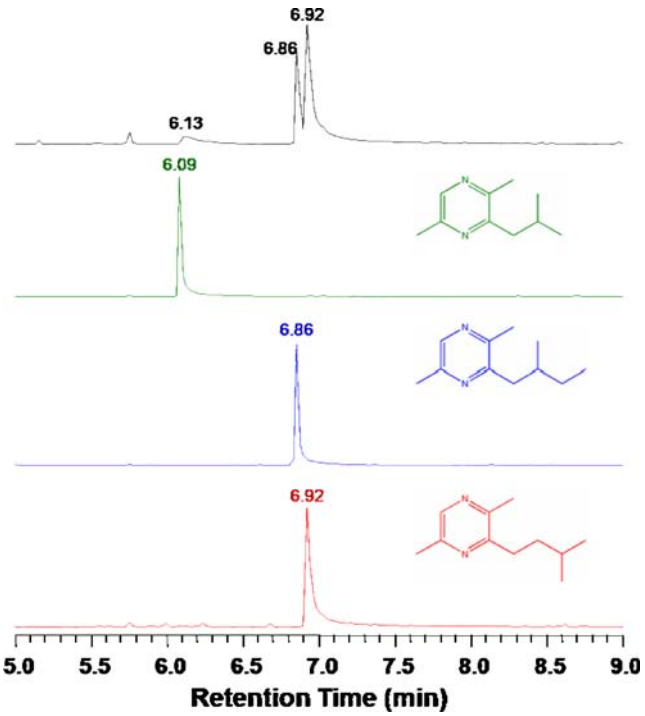


Fig. 3 GC-CI-MS total ion current chromatograms of natural *Phyllium westwoodii* defensive spray (black) compared with authentic synthetic standards: 3-isobutyl-2,5-dimethylpyrazine (top), synthetic (*S*)-2,5-dimethyl-3-(2-methylbutyl)pyrazine (middle), and synthetic 2,5-dimethyl-3-(3-methylbutyl)pyrazine (bottom). Each trace represents the injection into the GC apparatus of 4 μl of a sample made by extracting 2 μl of a D_2O NMR sample with 50 μl of methylene chloride (CH_2Cl_2). The NMR samples of synthetic reference compounds were 1 μl of pure neat material dissolved in 1 ml of D_2O

identified in the mixture using synthesis and gas chromatography (Fig. 3), mass spectrometry (Fig. 4), and NMR (Figs. 5, 6 and 7). This work demonstrates the increasingly apparent diversity of compounds produced by phasmid insects and the uniqueness of Phylliidae among the Phasmatodea.

Methods and Materials

Animal Material and Sample Collection A laboratory culture of *P. westwoodii* originated from Chiang Rai Province in northern Thailand, where the species thrives in tropical rain forest ecosystems. Rearing was conducted in a ventilated cage at a mean temperature of 23.5 °C, moderate humidity conditions, and a photoperiod of 12 h. Leaf insects were fed on *Quercus robur* and *Rubus ulmifolius*. To obtain the original sample of defensive spray (less than 1 µl), one adult female *P. westwoodii* was milked two times by placing a clean 1.5 ml glass vial over the glandular openings, and slightly agitating the insects. A second sample (approximately 2–4 µl), that consisted of seven milkings was obtained the same way from two adult females.

The smaller of the two samples mentioned above (less than 1 µl from a single female *P. westwoodii*) was dissolved in approximately 15 µl of D_2O and used for NMR analysis. Approximately 2 µl were removed from this sample after collection of NMR data, and extracted with CH_2Cl_2 for GC-MS analysis. Since it was unknown whether the compounds in the mixture contained exchangeable protons, an additional 2 µl sample was mixed with ~100 µl of H_2O and extracted with CH_2Cl_2 for GC-MS analysis.

Analytical Procedures NMR experiments were performed with a 600 MHz 1-mm triple resonance high temperature superconducting (HTS) cryogenic probe that was developed through collaboration between the University of Florida, the National High Magnetic Field Laboratory (NHMFL), and Bruker Biospin (Brey et al. 2006). Each sample was loaded into a 1-mm×100-mm capillary NMR tube (Norell, Inc.) with a 10 µl syringe with a removable 110-mm×30-gauge blunt needle. During NMR experiments, the capillary tube was held in a standard 10-mm spinner by using a Bruker MATCH™ device, and the capillary-MATCH-spinner combination was lowered vertically into the magnet on an air-column. The sample temperature was maintained at 20 °C. The spectrometer used for all NMR experiments was a Bruker Avance II 600. Additional NMR data acquisition parameters can be found in the Supplemental Material with their respective spectra. All data acquisition, processing, and analysis were done with Bruker TopSpin© 2.0 software. Chemical shift assignments were made by referencing the natural *P. westwoodii* ^{1}H and ^{13}C chemical shifts of the anomeric ^{1}H to 5.22 and ^{13}C to 94.8 ppm of alpha glucose based on the reported values for these resonances in the BMRB Metabolomic database (http://www.bmrb.wisc.edu/metabolomics/) (Ulrich et al. 2008). In order to verify the components in *P. westwoodii* defensive spray that were tentatively identified by

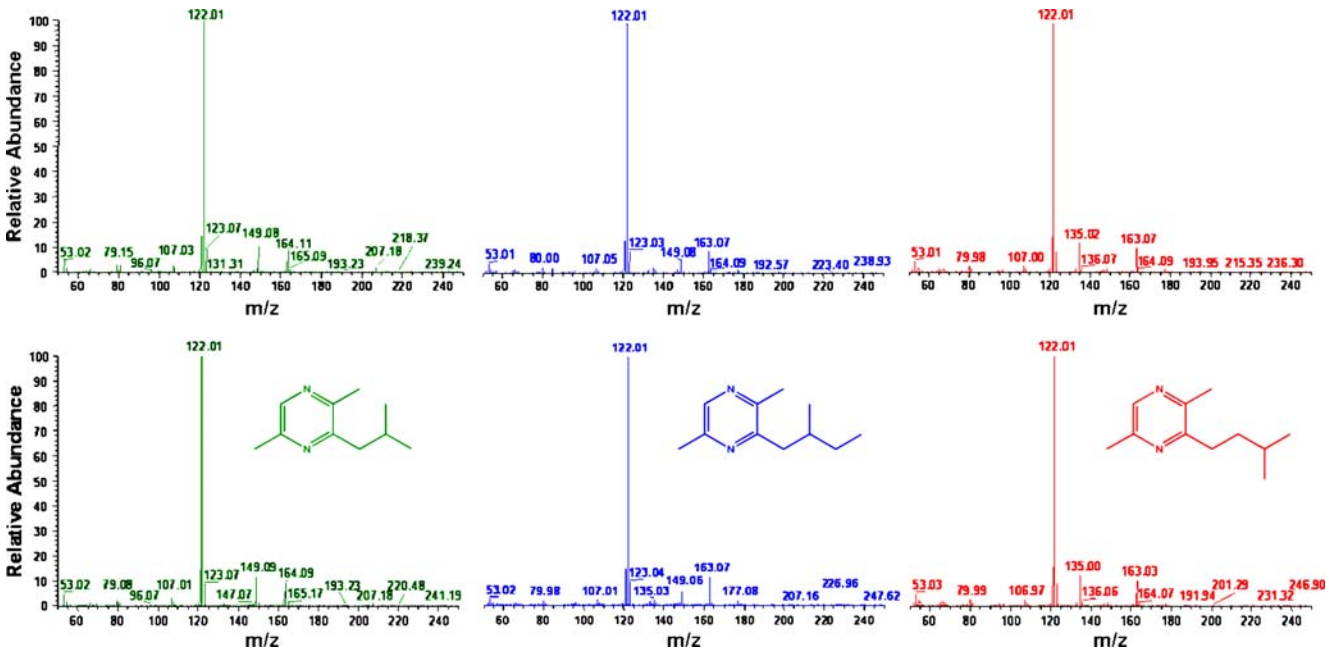


Fig. 4 Electron ionization mass spectra (GC-EI-MS) of compounds detected in *Phyllium westwoodii* defensive spray extracted with CH_2Cl_2 (methylene chloride) (top row; compare Fig. 3, peaks at 6.12, 6.86, and 6.92 min). Bottom row: Mass spectra from authentic synthesized standards

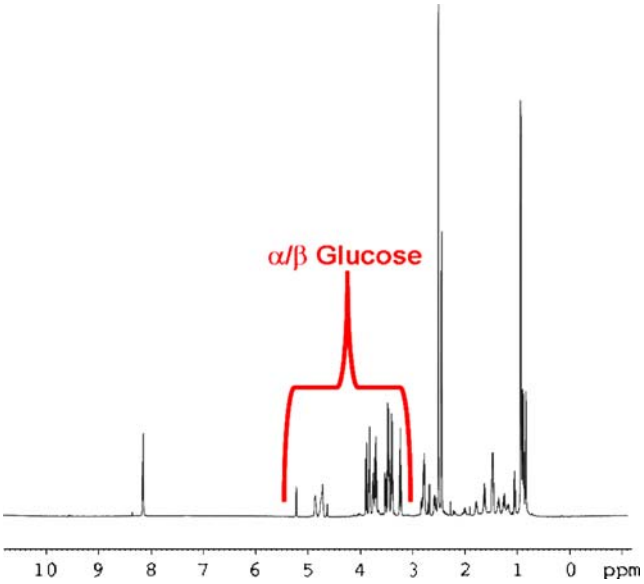


Fig. 5 1D ^{1}H NMR spectrum of *Phyllium westwoodii* defensive spray dissolved in deuterium oxide (D_2O). Resonances from glucose are shown under the bracket

GC-EIMS and to assign ^{1}H and ^{13}C chemical shifts to those compounds (Tables 1, 2 and 3), one- and two-dimensional (1D and 2D, respectively) datasets were recorded (Figs. 5, 6 and 7, and Supplemental Material Figs. S2–S5). Specifically, COSY, TOCSY, NOESY, and natural abundance ^{13}C HMQC and HMBC datasets were collected.

GC-MS analyses were performed with a Thermo Scientific Trace DSQ mass spectrometer, equipped with a Restek Rxi™-5 ms column (15 m×0.25 mm ID×0.25 um df) and with helium as carrier gas (flow rate=1 ml/min). Four µl of each sample were injected in splitless mode, for 2 min, followed by a split flow rate of 50 ml/min. The GC oven temperature was maintained at 40 °C for 3 min, and then increased at a rate of 20 °C/min to 260 °C. The injector and transfer line were set at 250 °C, while the ion source was at 180 °C. Compounds that eluted from the column underwent electron ionization (EI) at 70 eV, and ions from *m/z* 50 to 700 were detected. The electron-ionization mass spectra (EI-MS) of these peaks were used to query the NIST/EPA/NIH Mass Spectral Library of known compounds using the NIST MS Search (ver. 2) software (Stein et al. 1987–2002).

Synthesis of Pyrazines: General Procedure The Grignard reagent was prepared by slow addition of the appropriate alkyl bromide (1.0 equiv.) to a stirred mixture of magnesium turnings (1.2 equiv.) in dry ether (1.0 M) at room temperature. The resulting mixture was stirred for 2 h at room temperature, and then titrated with 2-hydroxybenzaldehyde phenylhydrazone (Love and Jones 1999). Commercial 3-chloro-2,5-dimethylpyrazine (1.0 equiv.) was added to a solution of Fe$(acac)_3$ (0.05 equiv.) in THF-NMP (9:1, 0.25 M), and the mixture was cooled to 0 °C in an ice-water bath. Grignard reagent (1.5 equiv) in ether was added dropwise to this solution. The resulting mixture was stirred for an additional 30 min, and then quenched by the addition of water. The aqueous layer was extracted with diethyl ether. The combined organic extracts were dried over $MgSO_4$, clarified with charcoal, filtered, and the solvent was removed under

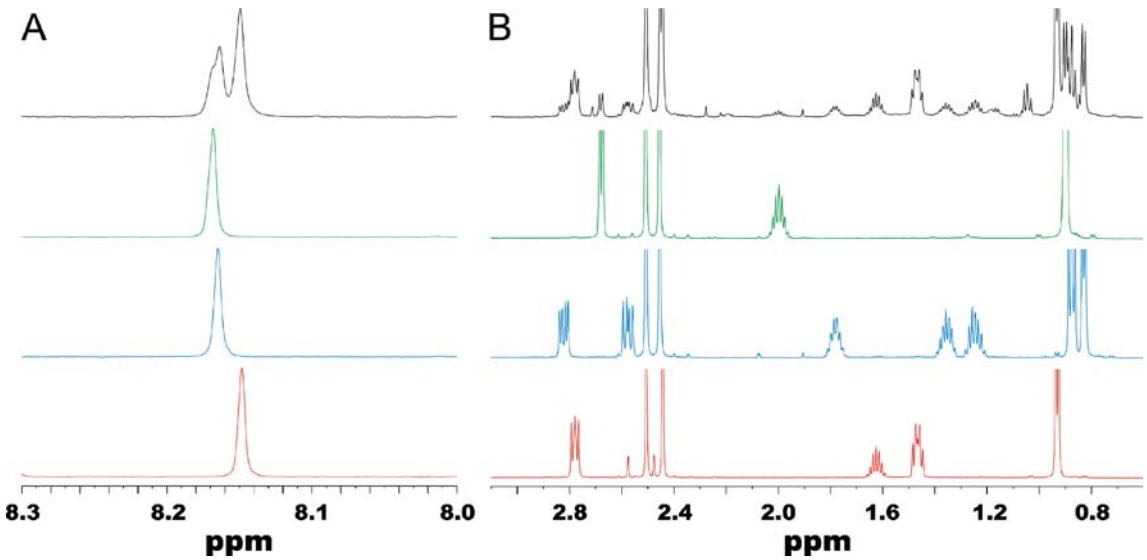


Fig. 6 1D ^{1}H spectra of natural *Phyllium westwoodii* defensive spray compared with authentic synthetic standards: **a** aromatic region and **b** aliphatic region. Spectra shown are of natural *P. westwoodii* defensive spray (top), synthetic 3-isobutyl-2,5-dimethylpyrazine (**1**) (2nd from top), synthetic (*S*)-2,5-dimethyl-3-(2-methylbutyl)pyrazine (**2**) (3rd from top), and synthetic 2,5-dimethyl-3-(3-methylbutyl)pyrazine (**3**) (bottom). All samples were analyzed in D_2O. The synthetic NMR samples were 1 µl of pure neat material dissolved in 1 mL of D_2O

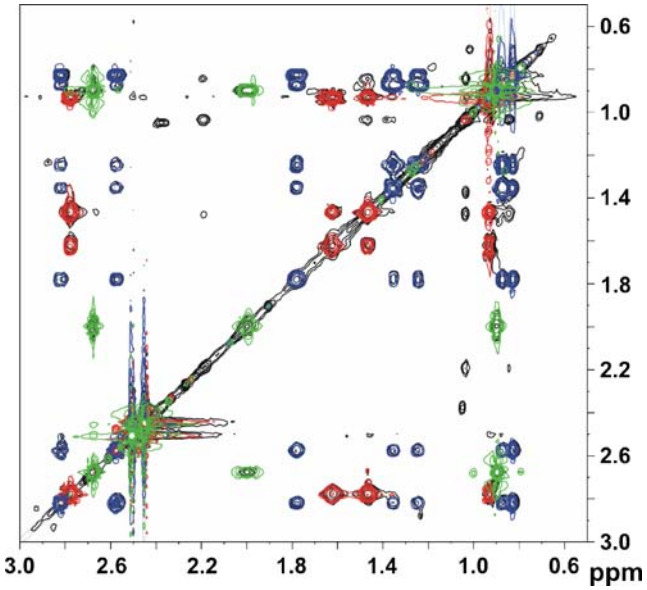


Fig. 7 Aliphatic sidechain region of 2D ^{1}H TOCSY spectra compared to authentic synthetic standards by overlaying the spectra. Spectra shown are of natural *Phyllium westwoodii* defensive spray (black), synthetic 3-isobutyl-2,5-dimethylpyrazine (**1**) (green), synthetic (*S*)-2,5-dimethyl-3-(2-methylbutyl)pyrazine (**2**) (blue), and synthetic 2,5-dimethyl-3-(3-methylbutyl)pyrazine (**3**) (red). All samples were analyzed in D_2O. The synthetic NMR samples were 1 μl of pure neat material dissolved in 1 ml of D_2O

reduced pressure. The crude product mixtures were purified by silica gel column chromatography, using a solution of 5% ethyl acetate in hexane.

3-Isobutyl-2,5-dimethylpyrazine (1) Yield: 42% (3.463 g, 21.08 mmol). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.94 (d, *J*= 6.8 Hz, 6 H), 2.12 (non, *J* = 6.8 Hz, 1 H), 2.48 (s, 3 H), 2.51 (s, 3 H), 2.65 (d, *J*=7.2 Hz, 2 H), 8.14 (s, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.3, 21.7, 22.6, 28.8, 43.7, 140.7, 149.0, 150.1, 154.4; IR (film): *v* 2957, 2869, 1450, 1370, 1288, 1168, 1074 cm−1; EI-MS 165.1386, HRMS-ESI (*m/z*): [M+] calcd for $C_{10}H_{16}N_2$ 165.1386, found 165.1390. Spectroscopic values for synthetic **1** were in agreement with reported literature values (Dickschat et al. 2005a).

(S)-2,5-Dimethyl-3-(2-methylbutyl)pyrazine (2) Yield. 46% (546 mg, 3.06 mmol). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.87 (d, *J*=6.8 Hz, 3 H), 0.92 (t, *J*=7.6 Hz, 3 H), 1.26 (m, 1 H), 1.42 (m, 1 H), 1.90 (m, 1 H), 2.49 (s, 3 H), 2.51 (s, 3 H), 2.56 (dd, *J*=8.4, 13.2 Hz, 1 H) 2.78 (dd, *J*=6.0, 13.2 Hz, 1 H), 8.13 (s, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 11.6, 19.0, 21.3, 21.6, 29.7, 34.9, 42.0, 140.7, 149.0, 150.1, 154.5; HRMS-ESI (*m/z*): [M+] calcd for $C_{11}H_{18}N_2$ 179.1543, found 179.1545, specific optical rotation: $[\alpha]_D^{25}$ = +8.8 (*c*=2.535, CH_2Cl_2). Spectroscopic values for synthetic **2** were in agreement with reported literature values (Dickschat et al. 2005a).

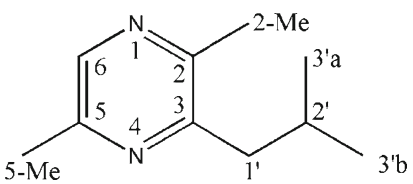
2,5-Dimethyl-3-(3-methylbutyl)pyrazine (3) Yield: 55% (2.458 g, 13.79 mmol). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.97 (d, *J*=6.8 Hz, 6 H), 1.53 (m, 2 H), 1.67 (sept, *J* = 6.8 Hz, 1 H), 2.47 (s, 1 H), 2.51 (s, 1 H), 2.75 (m, 2 H), 8.13 (s, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.2, 22.6, 28.5, 33.4, 37.8, 140.8, 148.5, 150.2, 155.3; IR (film): *v* 2956, 2927, 2870, 1536, 1452, 1320, 1278, 1254, 1169, 1080, 1034, 999, 942, 738; HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{11}H_{18}N_2$ 179.1543, found 179.1546. Spectroscopic values of synthetic **3** were in agreement with reported literature values (Dickschat et al. 2005b).

Results

In the GC-MS chromatogram of *Phyllium westwoodii* defensive spray, three main peaks were detected (Fig. 3). Two samples extracted from D_2O and H_2O preparations both showed nearly identical mass spectra, suggesting that they contained no exchangeable protons (Fig. 4 and data not shown).

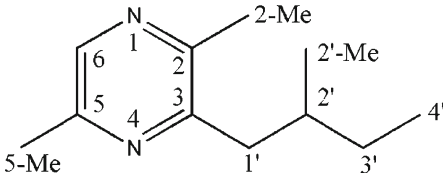
Mass spectra from all three peaks matched those of known alkyldimethylpyrazines with high scoring database

Table 1 NMR chemical shift assignments for synthetic **1** (3-isobutyl-2,5-dimethylpyrazine) dissolved in D_2O



Position	δ ^{1}H (ppm)	δ ^{13}C (ppm)	$J_{H\text{-}H}$ (Hz)
2	-	151.9	-
3	-	157.2	-
5	-	152.7	-
6	8.17	143.0	(s)
2-Me	2.51	22.6	(s)
5-Me	2.45	22.2	(s)
1’	2.68	45.3	7.46 (d)
2’	2.00	31.2	(m)
3’a/b	0.90	24.3	6.62 (d)

Table 2 NMR chemical shift assignments for synthetic **2** ((*S*)-2,5-dimethyl-3-(2-methylbutyl)pyrazine) dissolved in D_2O



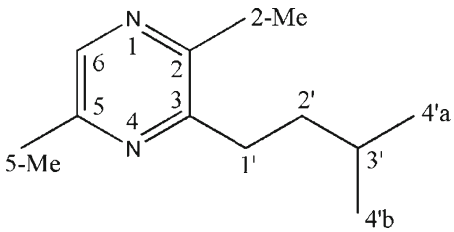
Position	δ ^{1}H (ppm)	δ ^{13}C (ppm)	$J_{H\text{-}H}$ (Hz)
2	-	152.3	-
3	-	157.8	-
5	-	153.0	-
6	8.16	143.0	(s)
2-Me	2.51	22.6	(s)
5-Me	2.45	22.18	(s)
1’	2.82	43.7	(m)
	2.58	-	(m)
2’	1.78	37.6	(m)
2’-Me	0.83	20.8	6.69 (d)
3’	1.36	31.9	(m)
	1.25	-	(m)
4’	0.88	13.5	7.44 (t)

hits (Fig. 4 and Supplemental Material Fig. S1) (Stein et al. 1987–2002). Additionally, the mass spectra match those of synthetic standards (Fig. 4). However, some isomers of the highest scoring compounds also were close matches (Supplemental Material Fig. S1). Additionally, similar mass spectra might be obtained from molecules with different relative placement of the alkyl chains and methyl groups on the pyrazine ring. Previous studies have shown phasmid defensive sprays to contain glucose (Dossey et al. 2006, 2007; Zhang et al. 2007), which is not directly amenable to study by GC-MS. Thus, NMR experiments were performed on both the natural defensive spray of *P. westwoodii* and synthetic reference standards in order to alleviate ambiguity in the identification of chemical components and to identify non-volatiles.

First, as with other phasmids, *P. westwoodii* defensive spray contained a significant amount of glucose: approximately 27% for one sample and 42% for an independent replicate sample of the total material observed by integration of the 1D ^{1}H NMR spectrum: anomeric resonances of glucose compared to the vinyl and ring methyl resonances for pyrazines (Fig. 5 and data not shown). Additionally, three sets of spin systems consistent with the aliphatic constituents of the alkyl pyrazines identified by GC-MS were the major peaks observed in 2D TOCSY NMR spectra (Fig. 7). However, considerable overlap was observed between some key resonances common to the three compounds (Fig. 6). Thus, all three substances were synthesized and examined in pure form in D_2O by NMR to verify their identity in the spectra of the natural product sample. 2,5-Dimethyl-3-alkylpyrazines **1**, **2**, and **3** were synthesized *via* iron-catalyzed cross-coupling (Furstner et al. 2002) of 3-chloro-2,5-dimethylpyrazine with the appropriate Grignard reagent, as described by Dickschat et al. (Fig. 8) (Dickschat et al. 2005a, b). Enantiopure (*S*)-(+)-1-bromo-2-methylbutane was used for formation of the Grignard reagent in the synthesis of **2** under the presumption that the pyrazine derives biosynthetically from *L*-isoleucine.

One-dimensional (1D) NMR spectra of the natural defensive mixture from *P. westwoodii* and the three synthetic pyrazines in D_2O are compared in Fig. 6. The

Table 3 NMR chemical shift assignments for synthetic **3** (2,5-dimethyl-3-(3-methylbutyl)pyrazine) dissolved in D_2O



Position	δ ^{1}H (ppm)	δ ^{13}C (ppm)	$J_{H\text{-}H}$ (Hz)
2	-	149.5	-
3	-	156.3	-
5	-	150.9	-
6	8.15	140.5	(s)
2-Me	2.51	20.1	(s)
5-Me	2.44	20.0	(s)
1’	2.78	32.6	(m)
2’	1.47	38.0	(m)
3’	1.63	28.2	6.6 (m)
4’a/b	0.93	22.1	6.71 (d)

alkyl and aromatic methyl region of the spectrum from 0.6–3.1 ppm (Fig. 6B) shows reasonably well-resolved resonances for the alkyl sidechains. A comparison of the spectrum of the mixture to those of the synthetic pyrazines in Fig. 6 indicates that the aromatic methyl resonances for all three compounds have nearly identical chemical shifts in the 2.4–2.5 ppm region. Additionally, the aromatic ^{1}H region at about 8.15 ppm shows at least three resonances, two of which almost completely overlapped. Thus, analysis of 2D spectra for the natural mixture and of the individual synthetic compounds was employed to resolve the ambiguity caused by the overlapping regions and obtain complete ^{1}H and ^{13}C chemical shift assignments for all three pyrazines.

Figure 7 shows the aromatic methyl and alkyl sidechain resonances of TOCSY spectra of the natural mixture (black) and the three synthetic pyrazines in color overlays (**1** green, **2** blue, and **3** red). Overlaying of two-dimensional NMR spectra can be a powerful and robust way to identify individual known substances in natural product mixtures. In the TOCSY spectrum of the mixture, three distinct alkyl spin systems were observed. For compound **1**, TOCSY correlations were observed between resonances at 2.68 ppm and the following: 2.00, and 0.90 ppm. COSY correlations were observed between resonances at 2.68 ppm and 2.00 ppm and between 2.00 ppm and 0.90, but not between 2.68 ppm and 0.90 ppm. For compound **2**, TOCSY correlations were observed between the resonance at 2.82 ppm and the following resonances: 2.58, 1.78, 1.36, 1.25, 0.88, and 0.83 ppm. Strong COSY correlations were observed between 2.82 and 2.58 ppm; 1.36 and 1.25 ppm; 1.36 ppm and 0.88 ppm; and 1.25 ppm and 0.88 ppm. Weaker COSY correlations are seen between the following resonances: 2.82 and 1.78; 2.58 and 1.78; 1.78 and 0.83 ppm. Oddly, no COSY ^{1}H-^{1}H correlations were observed in spectra from either natural or synthetic compound **2** between the methine 2′ resonance (1.78 ppm) and methylenes at 1.36 or 1.25 pm (Supplemental Material Fig. S3). However, distinct TOCSY correlations were observed between these pairs of resonances as expected (Fig. 7 and Supplemental Material Fig. S2). For compound **3**, TOCSY correlations were observed between 2.78 ppm and the following resonances: 1.47, 1.63, and 0.93 ppm. A strong COSY correlation was observed between the methylenes at 2.78 and 1.47 ppm and between the methyl and methylene resonances (1.47 and 0.93, respectively). However, only very weak COSY correlations were observed between the methylene resonance at 1.47 ppm and methyne at 1.63 ppm.

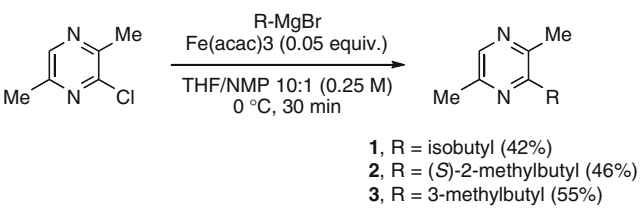


Fig. 8 Synthetic scheme for alkyldimethylpyrazines **1**–**3**

An overlapped set of spin systems with correlations between resonances at about 2.4–2.5 ppm and 8.15 ppm also were observed in TOCSY spectra. There were no clear ^{1}H-^{1}H correlations between those spin systems and the three alkyl ones (Fig. 7). However, the HMBC of the three pure synthetic pyrazines analyzed showed correlations between the 1′ ^{1}H and the 2 and 3 ^{13}C resonances (Supplemental Material Fig. S6). Additionally, the HMBC of the mixture showed a weak correlation between 2.78 and 158.5 ppm, demonstrating connectivity between the alkyl and other spin systems (Supplemental Material Fig. S5). Finally, in the HSQC experiment which was used, the signs (+/−) of the correlations were both sensitive to and consistent with the proposed methine, methylene, and methyl groups (Supplemental Material Fig. S4).

In addition to chemical analysis, a video was recorded which illustrates some features of the defensive behavior of *P. westwoodii* (Supplemental Material Fig. S7). This behavior is well established in the literature for other species in the same genus (Henry 1922; Bedford 1978; Zompro and Grösser 2003, and references therein). First, the insect was removed from the cage and placed on a stick for observation. This action sufficiently disturbed the insect causing it to freeze in a pose as if to play dead or possibly to resemble a leaf. The legs were positioned in a seemingly disordered conformation and were not all used to hold onto the stick. Next, when the offending stimulus was continued more aggressively, the insect compressed, became more active, attempted to escape, and produced stridulatory sounds by using its antennae. It is at this point that the insect released its defensive spray. Although the amount of secretion produced by *P. westwoodii* is small, to the human observer, its intense odor can be strongly perceived even at a distance of at least 30 cm. This odor was noticed only when *P. westwoodii* defended against attack or disturbance.

Discussion

These data demonstrate that the defensive spray of *Phyllium westwoodii* contains 3-isobutyl-2,5-dimethylpyrazine (**1**), 2,5-dimethyl-3-(2-methylbutyl)pyrazine (**2**), and 2,5-dimethyl-3-(3-methylbutyl)pyrazine (**3**), and α- and β-glucose. The release of this spray from *P. westwoodii* happens concurrently with a characteristic defense behavior (Supplemental Material Fig. S7 and described in the "Introduction") when approached by a human, which likely resembles an attack of natural predators of this species such as slow lorises and other primates (Francis Seow-Choen,

personal communication). Additionally, chemical sprays from homologous glands in other phasmid species have been shown experimentally to be effective in repelling potential predators such as ants, frogs, and birds (Eisner 1965; Carlberg 1985a, b, 1986, 1987; Chow and Lin 1986; Bouchard et al. 1997; Eisner et al. 1997). Thus, the prothoracic glandular spray from *P. westwoodii* is likely to be used predominantly for defense. However, it has not been shown if **1**, **2**, or **3** function as repellants of predators in the natural habitat of *P. westwoodii*.

Interestingly, these compounds are found widely in the aromas and flavors of foods that humans find pleasing (Maga and Sizer 1973). Some insects use these and other similar pyrazines as pheromones (Cavill and Houghton 1974; Cross et al. 1979; Blum 1981; Tengo et al. 1982). Also, the amount produced by *P. westwoodii* in response to being disturbed is small. Many phasmids produce only very small quantities of prothoracic glandular spray (Tilgner 2002), and the chemistry of their spray can vary over development (Dossey et al. 2008). Thus, it has been postulated that some species may use these substances for functions other than defense (Tilgner 2002; Dossey et al. 2008). It cannot be excluded that *P. westwoodii*, as well as other Phylliidae, use them for intraspecific communication. However, such a hypothesis is beyond the scope of this study and, as far as we are aware, has not yet been tested.

Other phasmid species produce monoterpenes (Meinwald et al. 1962; Smith et al. 1979; Chow and Lin 1986; Ho and Chow 1993; Bouchard et al. 1997; Dossey et al. 2006, 2007), a straight chain ketone (Schmeda-Hirschmann 2006), quinoline (Eisner et al. 1997), and other small secondary metabolites in their prothoracic defensive glands. However, this is the first report of pyrazines from these glands in any phasmid. In addition to *P. westwoodii* in this study, glucose also has been found in defensive sprays from other phasmids (Dossey et al. 2006, 2007; Zhang et al. 2007). Some Coleoptera of the family Chrysomelidae (leaf beetles) use precursors that are conjugated with glucose for monoterpene biosynthesis and transport into the defense glands (Feld et al. 2001; Laurent et al. 2003; Burse et al. 2007;Kunert et al. 2008). The common occurrence of glucose in insect defensive secretions, especially phasmids such as *P. westwoodii*, suggests that it may be similarly utilized in defensive secondary metabolite processing and/or transport. If so, this may represent a previously unreported mechanism for pyrazine production. This hypothesis requires further investigation.

The peculiar morphological features that characterize fossil and extant leaf insects (Zompro 2004; Wedmann et al. 2007) as well as the findings presented here, support the view that Phylliidae occupy a unique position among Phasmatodea. This study demonstrates yet another distinguishing feature of this unique group of phasmids. Many other phasmids produce monoterpenes or other secondary metabolites in their defensive spray, but the apparently homologous glands in *P. westwoodii* produce pyrazines, a class of compounds not previously reported from Phasmatodea. Pyrazines found in the chemical defense glands of *P. westwoodii* might even be considered an evolutionarily derived (apomorphic) feature of Phylliidae that further supports the monophyly of the taxon. The presence of glucose in walkingstick defensive sprays, along with the large size and ease of culture for these creatures, suggests their potential value as a model for the study of their biosynthetic pathways. Additionally, discovery of the functional role of pyrazines in *P. westwoodii* may shed new light on the role of secreted small molecules used by insects.

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Temperature and Food Availability Differentially Affect the Production of Antimicrobial Compounds in Oral Secretions Produced by Two Species of Burying Beetle

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Abstract Carrion beetles of the subfamily Nicrophorinae search and bury a carcass that they utilize for reproduction. After burial, the carcass is coated with oral secretions that delay its decomposition. Previously, oral secretions of *Nicrophorus marginatus* were found to show antimicrobial activity, whereas secretion of *N. carolinus* lacked significant activity. Here, we tested the effects of temperature, sex of the beetle, and food type on the antimicrobial properties of oral secretions of both species. Unlike previous findings, we found that oral secretions of *N. carolinus* had antimicrobial activity. Temperature had significant effects on the amount of secretion protein. When protein concentrations were standardized to 1 μ/ml, *N. marginatus* secretions had higher antimicrobial activity at cooler temperatures, while *N. carolinus* had higher activity at warmer ones. The sex of the beetle did not affect antimicrobial activity for either species. Beetles of both species that were fed whole rats contained more protein in their oral secretions than beetles fed with equally sized pieces of raw ground beef. After standardizing the resulting protein concentrations to 1 μg/ml, antimicrobial activity of oral secretion increased for *N. carolinus* after rat feeding, but not for *N. marginatus*. Our results highlight key ecological differences between these closely related species. In addition, they demonstrate the importance of experiments being conducted under varying environmental conditions when evaluating species for potential antimicrobial compounds.

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Keywords Carrion beetle · *Nicrophorus carolinus* · *Nicrophorus marginatus* · Temperature · Sex · Feeding · Antimicrobial secretion

Introduction

Insects come into contact with millions of microorganisms and rely mainly on their innate immunity to avoid infection from pathogens (Clarke 1973; Gupta 1979; Lamberty et al. 1999; Lavine and Strand 2002). In addition to avoiding individual infection, some insects also produce antimicrobial compounds that protect their eggs or limit decay of carrion or dung that they use for reproduction. For example, the termite *Pseudacanthotermes spiniger* depends upon a symbiotic fungus for nutrition; the fungus grows on the fecal material of the termites. The termites coat their eggs with saliva that contains antimicrobial peptides, and this protects their eggs from the fungi (Lamberty et al. 2001). Another example is that of the European beewolf that applies the contents of its postpharyngeal gland to its prey of paralyzed honeybees where it delays fungal infestation (Strohm et al. 2008).

Carrion beetles (Coleoptera: Silphidae) also have an antimicrobial oral secretion (Hoback et al. 2004). Silphid beetles respond to carrion that they externally digest by secreting extracellular enzymes produced in the salivary gland (Ratcliffe 1996; Rana et al. 1997). Members of the subfamily Nicrophorinae often are referred to as 'burying beetles' because they arrive at the carcass during the early stages of decomposition and then bury and tend the carcasses (Milne and Milne 1976; Ratcliffe 1996). After interment, burying beetles coat the carcass with secretions that contain antimicrobial activity that delays the decom-

position of the carcass ensuring a reproductive source (Milne and Milne 1976; Ratcliffe 1996; Scott 1998; Hoback et al. 2004).

Burying beetles exhibit a unique form of bi-parental care where a male and female pair tend the carcass (Scott 1998). When a suitable carcass is found, a pair of beetles bury the carcass 10 cm or more deep by excavating underneath the carcass until it is completely covered (Ratcliffe 1996). Once buried, the pair will strip it of fur or feathers and shape it into a ball, known as the brood ball (Ratcliffe 1996; Trumbo 1996). Once the brood ball is formed, the beetles coat the outside with oral and/or anal secretions that help control decomposition.

Carcasses that were treated by silphid beetles were less contaminated by mold than those that were untreated, even after the beetles were removed (Suzuki 2001). This suggests that the chemical effects of the secretion remain active when no beetles are present. Rana et al. (1997) found the oral secretion of *Nicrophorus marginatus* to contain phospholipase A_2 (PLA_2). PLA_2 is responsible for the pre-oral hydrolyzing of fatty acids. The oral PLA_2 may preserve the carcass by directly attacking bacterial membranes (O'Leary 1962).

In a study of 10 species of *Nicrophorus* beetles from Nebraska, antimicrobial activity was found in the oral secretions of all but 2 species (Hoback et al. 2004). The results showed that the secretions of *N. orbicollis, N. americanus, N. obscurus, N. tomentosis,* and *N. marginatus* exhibited antimicrobial properties that significantly reduced the amount of bacteria by 20 to 40%. The antimicrobial activity worked against both gram-positive and gram-negative bacteria, and it appeared to be proteinaceous as its activity was diminished by heat and proteinase treatment (Hoback et al. 2004). Oral secretions of *N. pustulatus* and *N. carolinus* failed to reduce the number of bacteria.

The lack of antimicrobial activity in the secretion of *N. pustulatus* may be explained by its reproductive strategy, which is to parasitize the brood ball of another species (Wilson and Knollenberg 1984). The lack of antimicrobial activity in the oral secretion of *N. carolinus* is more difficult to explain and does not appear to correlate with size, habitat association, or phylogeny. However, Hoback et al. (2004) did not control for beetle sex when collecting secretions, nor did they directly test the effects of environmental temperature or type of carrion on secretion activity.

In an attempt to elucidate conditions that may explain the lack of antimicrobial activity of oral secretion from *N. carolinus* in our earlier study, we examined the effects of the temperature at which the beetles are held, the sex of the beetle, and the food type given to the beetle on the antimicrobial properties of saliva from *N. carolinus* and *N. marginatus*.

Methods and Materials

Insect Collection During the summers of 2005 and 2007, adult *N. marginatus* and *N. carolinus* beetles were collected in Kearney and Lincoln Counties in Nebraska, U.S.A., using baited pitfall traps, as outlined in Bedick et al. (1999). These traps consisted of 5 gallon (21 l) buckets that were baited with rotted jumbo rats purchased from RodentPro.com. Beetles were collected each morning before 10:00 a.m., and rats were replaced every 3 d. Beetles were identified according to Ratcliffe (1996) and transported to the laboratory at the University of Nebraska at Kearney.

Secretion Collection When carrion beetles are harassed, most will excrete oral and anal secretions, while some produce defensive anal sprays (Rana et al. 1997; Scott 1998). Secretions were collected by placing a sterile cotton swab between the mandibles, while applying gently pressure to the abdomen. An individual sample consisted of oral secretion from 10 beetles placed into 1 ml of 0.1 M Tris buffer at pH 7, a variation of the protocol of Hoback et al. (2004). Samples were centrifuged at 10,000 rpm for 1 min, and the supernatant was collected. Secretions were sterilized by using a 0.22 μm syringe filter with a 0.80 μm pre-filter (Pall Corporation, Ann Arbor, MI, USA) and stored at −20°C until assays were conducted.

Protein Quantification The concentration of protein in the secretions was quantified with BCA Protein Assay Kits (Pierce Biotechnology, Inc., Rockford, IL, USA) using bovine serum albumin as a standard. Samples were read at 562 nm with a Beckman DU-640 spectrophotometer (Beckman Coulter, Fullerton, CA, USA) with distilled water as a blank. Protein content from each sample was standardized to 1.0 μg/ml before toxicity analysis was conducted.

Statistical analysis for the temperature samples was conducted using SigmaStat 3.1 (Systat Software, Inc.). Means were tested with Kruskal-Wallis *ANOVA* followed by Dunn's Method for mean separation ($P<0.05$). For the effects of sex and food type on protein content, SigmaStat's Student *t*-test was used ($P<0.05$).

Toxicity Analysis Samples were assayed with a Microtox Model 500 Analyzer (SDI, Newark, DE, USA). The Microtox quantifies the survival of the bacterium, *Vibrio fisherii,* by measuring decrease in bioluminescence associated with cell death. All samples were standardized to 1 μg/ml protein concentration and assayed in triplicate with the 2% screening test, following the procedure in the MicrotoxOmni™ Software (AZUR Environmental, Newark, DE, USA) using 10.0 μl of 0.1 M Tris buffer at pH 7 as a control. Statistical analysis was done by using an

Table 1 Protein content (mean±se) of the oral secretion of *Nicrophorus carolinus* and *N. marginatus*

Protein (μg/ml)					
N. carolinus					
Temperature[a]	4°C	10°C	15°C	25°C	30°C
	12.8±0.73a	23.1±0.78ab	34.9±3.36b	41.8±1.18b	29.1±0.39ab
Sex[b]	Male	Female			
	12.53±1.93a	14.54±2.04a			
Food Type[c]	Ground beef	Rat			
	5.62±1.02a	22.11±5.24b			
N. marginatus					
Temperature[a]	4°C	10°C	15°C	25°C	30°C
	55.6±9.20a	44.9±7.58ab	25.3±4.41bc	20.6±0.84bc	12.0±3.43c
Sex[b]	Male	Female			
	15.07±1.97a	17.58±2.64a			
Food Type[c]	Ground beef	Rat			
	4.18±0.28a	19.15±6.23b			

[a] Number of replications: $N=6$ for *N. marginatus* and $N=4$ for *N. carolinus* run in triplicate. Difference of means were tested using Kruskal-Wallis analysis of variance, followed by a Dunn's test when significant differences were detected ($P<0.05$). Different letters represent a significant difference between means.

[b] Number of replications: $N=14$ for *N. marginatus* and $N=5$ for *N. carolinus* run in triplicate. Difference of means were tested using Student t-test ($P<0.05$). Different letters represent a significant difference between means.

[c] Number of replications: $N=6$ for *N. marginatus* and $N=4$ for *N. carolinus* run in triplicate. Difference of means were tested using Student t-test ($P<0.05$). Different letters represent a significant difference between means.

ANOVA followed by a Tukey Test when differences were detected ($P<0.05$).

Temperature Assay Beetles were maintained as Hoback et al. (2004) by separating species and housing them in plastic containers with moistened soil from their habitat. Beetles were placed in one of five environmental chambers set at 4°, 10°, 15°, 25°, and 30°C, respectively, with a 15/9 h light/dark cycle. When beetles encounter a whole carcass, a suit of behaviors is initiated where an individual male and female fight off other beetles and bury the carcass for use in reproduction (Milne and Milne 1976). To standardize food availability and feeding status while examining the effects of temperature, we used commercially available raw 85% lean ground beef. Ground beef was weighed and fed to beetles daily. Any uneaten ground beef was removed daily.

Oral secretions were collected after 3 d in the environmental chambers. For each temperature at which beetles were kept, 6 samples of *N. marginatus* were collected and 4 samples of *N. carolinus.* All assays were conducted in triplicate. Once 3-d-incubation was complete, all beetles were released so that no beetle would be housed in the laboratory more than 3 d.

Sex Assay Adult beetles were sexed following the protocol of Trumbo (1996). The oral secretion was collected immediately, and the beetles released. For *N. marginatus*,

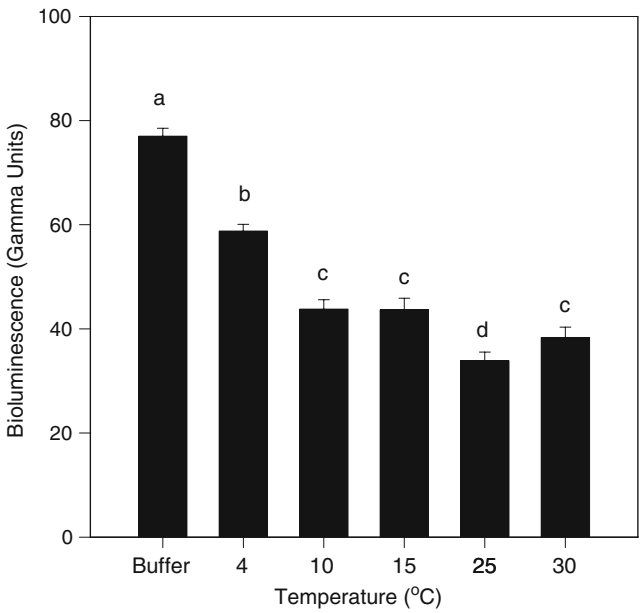

Fig. 1 Antimicrobial activity of oral secretion from *Nicrophorus carolinus* when maintained at five temperatures as compared to a Tris only buffer sample. Antimicrobial activity was measured using the Microtox 500 Analyzer which measures the bioluminescence in Gamma Units of *Vibrio fischerii*. Plotted are the mean + SE of $N=3$ experiments measured in triplicate. Data were statistically analyzed using ANOVA followed by a Tukey test when significance was detected ($P<0.05$). Different letters represent a significant difference between bars

a total of 14 secretion samples of each sex was collected and tested for antimicrobial activity. For *N. carolinus*, a total of 5 secretion samples was collected and tested for activity. All assays were conducted in triplicate.

Food Differences Beetles were separated into groups of 15 (both male and female) and placed into 30 X 15 X 20 cm glass containers containing moistened soil from their habitat. As a food source, beetles either were given a freshly thawed large rat at room temperature or raw 85% lean ground beef (Hamburger) that weighed approximately the same as the rat. All experiments were conducted at room temperature (21–24°C) in a fume hood to reduce odor. Secretions were collected after 3 d. A total of 6 secretion samples were collected from *N. marginatus* and tested for antimicrobial activity. For *N. carolinus*, a total of 4 secretion samples were collected. All assays were conducted in triplicate. Once the 3-d-incubation was complete, all beetles were released so that no beetle would be housed in the laboratory more than 3 d.

Results

Temperature Assays Our assays on the effect of temperature on protein content of oral secretion of *N. carolinus* revealed that beetles kept at 4°C produced significantly less protein than the 15°C and 25°C samples ($P<0.05$) (Table 1). After standardizing the protein content to 1.0 µg/ml, antimicrobial activity was quantified using the Microtox assay. *N. carolinus* oral secretions significantly inhibited growth compared to the buffer only control at all temperatures (Fig. 1). The highest antimicrobial activity was found for samples from beetles held at 25°C, with a 56% reduction in bioluminescence, significantly higher than samples from all other temperatures ($P<0.05$). The lowest antimicrobial activity was found in the 4°C sample with a 23.7% reduction in bioluminescence.

Protein content of oral secretions of *N. marginatus* was highest when beetles were kept at 4°C and lowest in the 30°C samples (Table 1). After standardizing protein content, all oral secretion samples had significantly reduced bacterial growth as compared to the buffer only control ($P<0.05$). The oral secretions with the greatest activities were collected from the beetles held at 4°C and 10°C with reduction of bioluminescence of 67.7% and 64.83%, respectively. These samples were not significantly different from each other, but they were significantly different from all other samples taken from beetles kept at other temperatures (Fig. 2).

Sex Assays No differences ($P>0.05$) were found between protein content of male and female oral secretions for either species (Table 1). The protein content from the oral secretions isolated from *N. marginatus* was slightly, but not significantly higher than of *N. carolinus.* No significant differences ($P>0.05$) were found between male and female antimicrobial activity from oral secretions in either species, but antimicrobial activity from oral secretions in both sexes was significantly different from the controls with buffer only (Fig. 3a and b).

Food Differences Oral secretion of rat-fed *N. carolinus* beetles contained 3.93 times more ($P<0.05$) protein than the secretion of the ground beef-fed beetles (Table 1). After standardization, the ground beef (Hamburger)- and rat-fed samples exhibited significantly different ($P<0.05$) antimicrobial activity as compared to the buffer control, but they did not differ from each other (Fig. 4a).

Oral secretion of *N. marginatus* contained 4.59 times more ($P<0.05$) protein than secretion of the ground beef-fed beetles (Table 1). After standardization of protein content, the antimicrobial activity of the oral secretions from *N. marginatus* given rat was significantly different ($P<0.05$) from both the buffer control and the ground beef (Hamburger)-fed samples, but samples from the ground beef-fed beetles were not significantly different from the buffer only control (Fig. 4b).

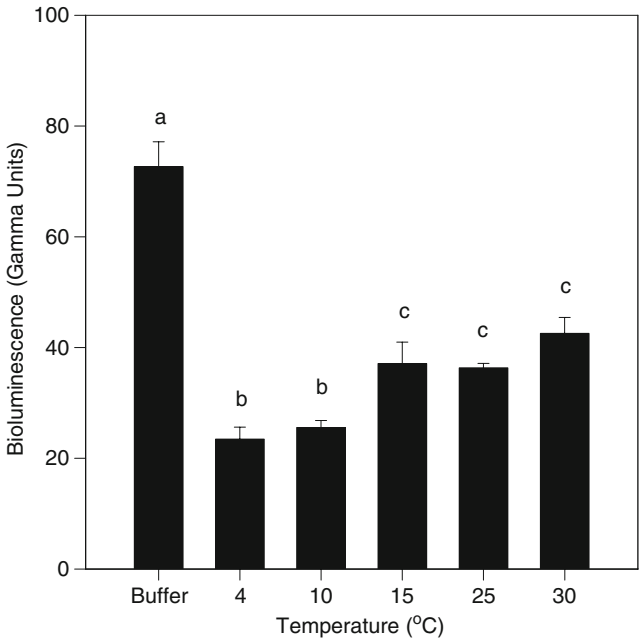

Fig. 2 Antimicrobial activity of oral secretion from *Nicrophorus marginatus* when maintained at five temperatures as compared to a Tris only buffer. Antimicrobial activity was measured using the Microtox 500 Analyzer which measures the bioluminescence in Gamma Units of *Vibrio fischerii*. Plotted are the mean + SE of $N=3$ experiments measured in triplicate. Data were statistically analyzed using ANOVA followed by a Tukey test when significance was detected ($P<0.05$). Different letters represent a significant difference between bars

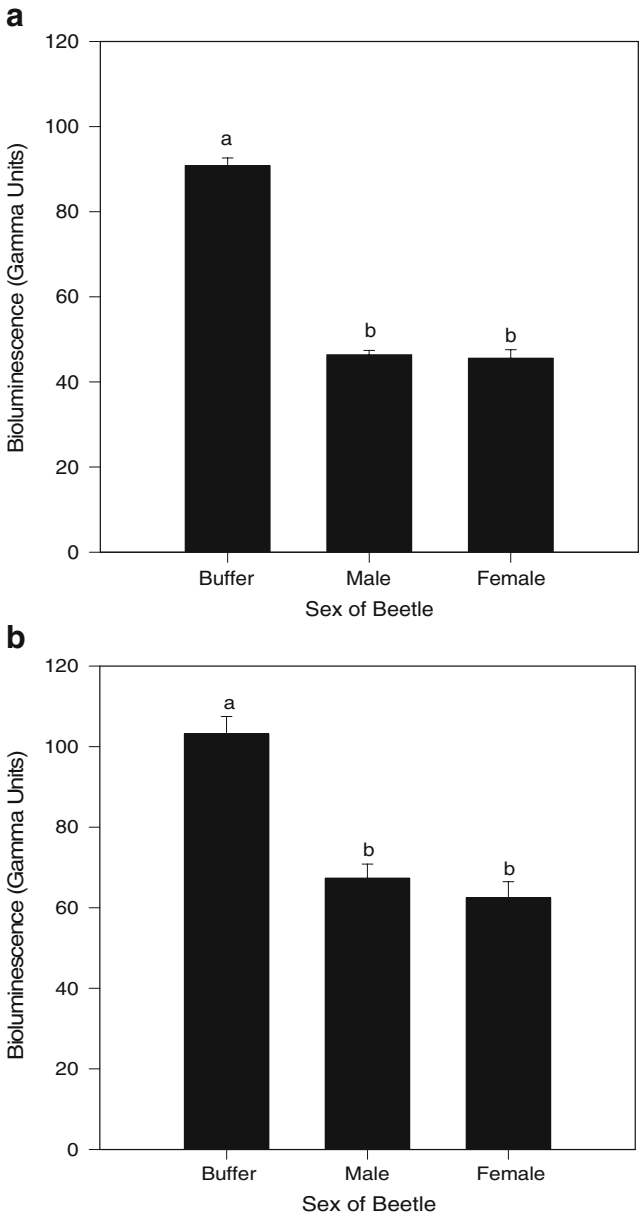

Fig. 3 Antimicrobial activity of oral secretion from male and female *Nicrophorus carolinus* (3**a**) and *N. marginatus* (3**b**) beetles as compared to a Tris only buffer. Antimicrobial activity was measured using the Microtox 500 Analyzer which measures the bioluminescence in Gamma Units of *Vibrio fischerii*. Plotted are the mean + SE of $N=3$ experiments measured in triplicate. Data were statistically analyzed using ANOVA followed by a Tukey test when significance was detected ($P<0.05$). Different letters represent a significant difference between bars

Discussion

Two environmental conditions were identified that influence the production of antimicrobial proteins found in oral secretion. Secretions from beetles of *N. carolinus* contained more protein and exhibited greater antimicrobial activity when beetles were kept at higher temperatures (25°C) (Table 1; Fig. 1), while the secretion of *N. marginatus* contained more protein and exhibited greater antimicrobial activity when beetles were kept at lower temperatures (4–10°C) (Table 1; Fig. 2). Although sex of the beetles did not influence antimicrobial activity of the secretion for

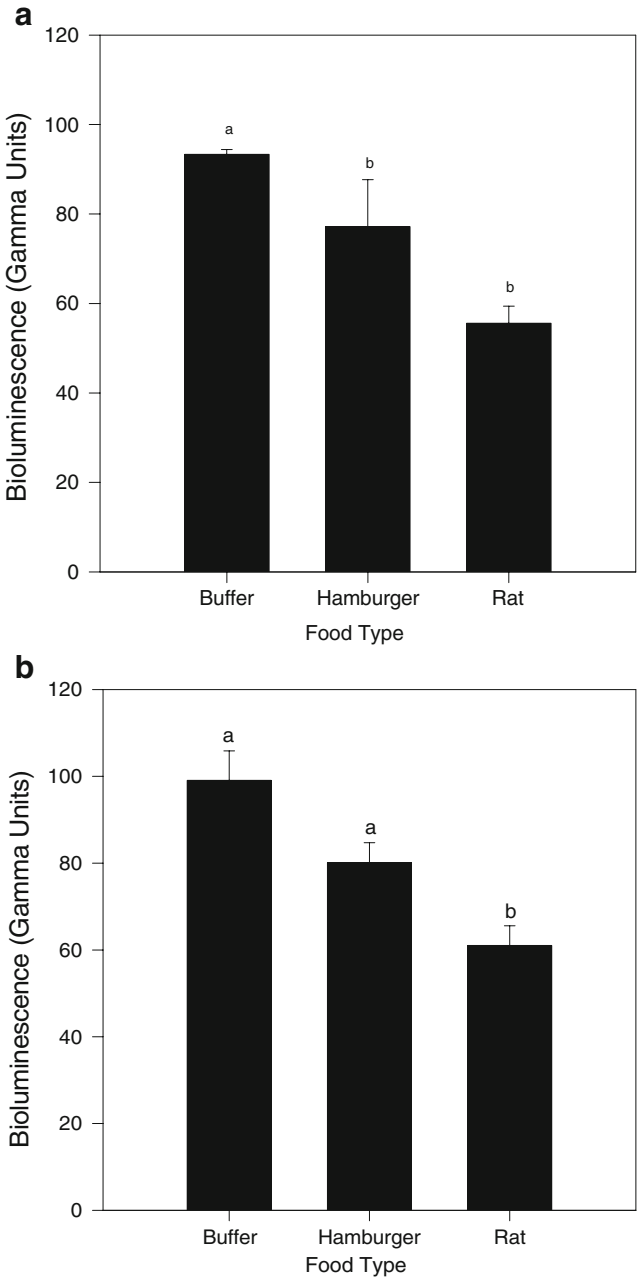

Fig. 4 Antimicrobial activity of oral secretion from *Nicrophorus carolinus* (4**a**) and *N. marginatus* (4**b**) beetles using two different food types (rat; Hamburger=ground beef) as compared to a Tris only buffer. Antimicrobial activity was measured using the Microtox 500 Analyzer which measures the bioluminescence in Gamma Units of *Vibrio fischerii*. Plotted are the mean+SE of $N=3$ experiments measured in triplicate. Data were statistically analyzed using ANOVA followed by a Tukey Test when significance was detected ($P<0.05$). Different letters represent a significant difference between bars

either species, food type altered antimicrobial activity of the secretion. Both species had significantly more protein in their oral secretions when provided with a rat as compared to ground beef (Table 1). The antimicrobial activity of the secretion also increased for *N. marginatus* fed on rats, but *N. carolinus* activity did not increase (Fig. 4). These data indicate that not only environmental conditions influence antimicrobial activity of the oral secretion, but different species also vary in their responses to these conditions.

Nicrophorus beetles generally become active when nighttime temperatures are above 13°C (Trumbo 1996). The reproductive season of the two species tested in this study almost completely overlap. In Nebraska, the active season of *N. marginatus* begins in February and ends in October. The active season of *N. carolinus* also ends in October, but activity begins in March (Ratcliffe 1996). According to the National Weather Service (2006), the average temperature for February 2006 was −2.2°C and 2.6°C for March 2006. Because *N. marginatus* begin their active season when the temperature is cooler, the optimum temperature of reproduction for *N. marginatus* may be lower than that of *N. carolinus*.

Hoback et al. (2004) found the antimicrobial activity of the oral secretions of burying beetles to be proteinaceous in nature, and protein activity is dependent upon temperature (Somero 1978; and Jaenicke et al. 1990). Antimicrobial activity in the oral secretion of *N. carolinus* was highest when beetles were kept at 25°C (Fig. 1), and the protein concentration was greatest for the 25°C samples, but only significantly greater than the 4°C samples (Table 1). These data also are supported by the ecology of *N. carolinus*. In Nebraska, *N. carolinus* is most common in the western part of the state in sandier soils (Anderson and Peck 1985; Ratcliffe 1996; Bishop et al. 2002). This environment would be hotter and drier than the preferred habitat of *N. marginatus*, which uses loam soils.

All of the samples for *N. marginatus* exhibited antimicrobial activity, with the 4°C and 10°C samples exhibiting the highest activity. As the temperature increased, a decrease in antimicrobial activity was observed (Fig. 2). The protein content of the oral secretion of *N. marginatus* also showed that the 4°C and 10°C samples had significantly more protein than the 30°C sample (Table 1). From the current findings, the antimicrobial activity of the beetles' oral secretions is most active at temperatures below 20°C. Bedick et al. (2006) reported a 50% mortality rate due to water loss for *N. marginatus* beetles when kept above 20°C in low humidity conditions for 10 h.

Parental supply of antimicrobial oral secretion is essential for larval success. Both parents are equally capable of producing antimicrobial oral secretion. In our experiment, beetles were collected, they were sexed, and their oral secretions were collected on the same day. Neither species showed significant differences between the antimicrobial activity or the protein content of the oral secretion produced by male or female beetles (Fig. 3; Table 1). This supports the findings of Scott (1989) who reported no differences in number, total weight, or survival of larvae when reared with single silphid males or females.

Because carrion is a rare resource (Eggert and Muller 1992), beetles cannot be selective, and numerous types of carrion have been found to attract burying beetles to traps (Bedick et al. 1999). The secretions of *N. carolinus* fed by either food type showed no difference in antimicrobial activity, and oral secretions of beetles fed by both food types showed significant antimicrobial activity (Fig. 4a). However, the secretions of *N. marginatus* showed a significant difference in antimicrobial activity when fed ground beef as compared to carrion. Although food type did not significantly affect antimicrobial activity of the oral secretion of *N. carolinus*, a significantly greater amount of protein was found in oral secretions from individuals fed rat carcasses than in secretions of those fed ground beef. The same was true for *N. marginatus*. The oral secretion from the rat fed beetles contained more than twice the amount of protein than the secretions from the ground beef fed beetles (Table 1). By producing larger quantities of protein, beetles would likely be able to protect successfully a brood ball from decomposition, even if the activity of the secretion does not change.

The results of this study indicate the importance of studying insects and potentially other groups under a variety of experimental conditions when investigating antimicrobial activity of their secretions. Temperature and carrion both were linked with changes in protein content of oral secretions of two carrion beetle species, but temperature also changed antimicrobial activity of the secretions. These factors are important in understanding under which conditions these beetles will be able to successfully reproduce. Other factors in the environment and beetle biology may also play a role in changing antimicrobial activity of the secretions, and these differences may be seen with other *Nicrophorus* species.

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Bacterial Attraction and Quorum Sensing Inhibition in *Caenorhabditis elegans* Exudates

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Abstract *Caenorhabditis elegans*, a bacterivorous nematode, lives in complex rotting fruit, soil, and compost environments, and chemical interactions are required for mating, monitoring population density, recognition of food, avoidance of pathogenic microbes, and other essential ecological functions. Despite being one of the best-studied model organisms in biology, relatively little is known about the signals that *C. elegans* uses to interact chemically with its environment or as defense. *C. elegans* exudates were analyzed by using several analytical methods and found to

Author contributions Fatma Kaplan and Dayakar V. Badri contributed equally and led the study; Fatma Kaplan, Aaron T. Dossey, Ramadan Ajredini, Hans Alborn and Michael Stadler intellectually contributed to the worm exudate protocol which was developed in the ASE laboratory; Fatma Kaplan, Ramadan Ajredini, and Rathika Nimalendran collected exudates; Dayakar V. Badri conducted bacterial bioassays; Hans Alborn collected LC-MS data; Fatma Kaplan and Cherian Zachariah collected NMR data; Fengli Zhang, Steven L. Robinette and Rafael Brüschweiler did COLMAR analysis; Fatma Kaplan, Cherian Zachariah, Michael Stadler, and Aaron T. Dossey manually analyzed NMR data. Sanja Roje and Francisco Sandoval analyzed amino acids by HPLC; Lanfang H. Levine analyzed young adult exudates by GC-MS; Wei Zhao did principle component analysis; Fatma Kaplan, Dayakar V. Badri, Arthur S. Edison and Jorge M. Vivanco analyzed the data and wrote the paper with help from the entire team.

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contain 36 common metabolites that include organic acids, amino acids, and sugars, all in relatively high abundance. Furthermore, the concentrations of amino acids in the exudates were dependent on developmental stage. The *C. elegans* exudates were tested for bacterial chemotaxis using *Pseudomonas putida* (KT2440), a plant growth promoting rhizobacterium, *Pseudomonas aeruginosa* (PAO1), a soil bacterium pathogenic to *C. elegans*, and *Escherichia coli* (OP50), a non-motile bacterium tested as a control. The *C. elegans* exudates attracted the two *Pseudomonas* species, but had no detectable antibacterial activity against *P. aeruginosa*. To our surprise, the exudates of young adult and adult life stages of *C. elegans* exudates inhibited quorum sensing in the reporter system based on the *LuxR* bacterial quorum sensing (QS) system, which regulates bacterial virulence and other factors in *Vibrio fischeri*. We were able to fractionate the QS inhibition and bacterial chemotaxis activities, thus demonstrating that these activities are chemically distinct. Our results demonstrate that *C. elegans* can attract its bacterial food and has the potential of partially regulating the virulence of bacterial pathogens by inhibiting specific QS systems.

Keywords *C. elegans* exudates · Bacterial chemotaxis · Metabolomics · Quorum sensing inhibitor · Chemical ecology · Nematodes

Introduction

Caenorhabditis elegans was the first metazoan to have its genome sequenced (Wilson et al. 1994), and has one of the best annotated animal genomes (www.wormbase.org). Its entire cell lineage from a single fertilized egg to an adult is known and has been related to the animal's anatomy (Sulston et al. 1983), and its anatomical ultrastructure has been described comprehensively by thin-section electron microscopy (White et al. 1986). *C. elegans* is particularly tractable for genetic studies (Brenner 1974), and as a result many signal transduction pathways have been identified. Several biomedically important discoveries have been made using *C. elegans* that include apoptosis (Ellis et al. 1991; Yuan et al. 1993), RNAi (Fire et al. 1998), and the *in vivo* application of the green fluorescent protein (Chalfie et al. 1994). In short, *C. elegans* is one of the best-understood animals in science. Natural *Caenorhabditis* species can be isolated from compost heaps, soil, decaying fruits, and even carrier invertebrates (Barriere and Felix 2006). Despite the wealth of genetic, cellular, and anatomical information, relatively little is known about *C. elegans* in its natural environment.

Small molecules are the primary mode of communication for most organisms. Bacteria, fungi, and plants release a variety of chemicals that attract or deter other organisms (Adler 1966; Mesibov and Adler 1972; Adler et al. 1973; Tso and Adler 1974; van der Drift et al. 1975; Hedblom and Adler 1983; Lugtenberg et al. 1999; Singh and Arora 2001; Bacilio-Jiménez et al. 2003; Kumar et al. 2007). Plant roots produce exudates that are capable of killing microbes, and these antimicrobial exudates can be up-regulated in response to pathogens (Walker et al. 2004; Bais et al. 2005). Several species of bacteria release acyl homoserine lactones to determine their local cell density (quorum sensing—QS), activate virulence factors at high cell density, and aggregate into biofilm (Miller and Bassler 2001; Williams et al. 2007). Some bacterial QS signals have been shown to attract *C. elegans* (Beale et al. 2006). Some species of plants (Givskov et al. 1996; Persson et al. 2005) and fungi (Rasmussen et al. 2005b) produce compounds that inhibit bacterial QS. Similar to bacteria, *C. elegans* also sense their own population density *via* dauer pheromones (Golden and Riddle 1982), which are ascaroside sugars with modified fatty acid groups (Jeong et al. 2005; Butcher et al. 2007, 2008; Srinivasan et al. 2008). These pheromones regulate the formation of a specific larval stage called dauer by integrating chemical cues from both the pheromone and an unknown chemical cue from their bacterial food source (Golden and Riddle 1982). *C. elegans* hermaphrodites also produce a chemical cue that attracts males (Simon and Sternberg 2002; White et al. 2007), and this mating cue recently was discovered to consist of a synergistic mix of known dauer pheromones called ascr#2 and ascr#3 (Butcher et al. 2007) and a glycosylated analogue called ascr#4, which is active at concentrations much lower than that required for dauer formation (Srinivasan et al. 2008). Recently, additional *C. elegans* ascarosides have been discovered with both dauer and mating activities (Pungaliya et al. 2009). Despite extensive recent research into *C. elegans* chemical signaling, relatively little is known about how *C. elegans* chemically interacts with microorganisms in its environment or even its natural food (Avery and Shtonda 2003; Shtonda and Avery 2006).

In this study, we present a comprehensive analysis of the chemical composition of *C. elegans* exudates by using several analytical techniques, which allowed the identifica-

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tion of several amino acids, sugars, and organic acids that are released by the nematode into its environment. The majority of these chemicals were amino acids, and we present a quantitative analysis of changes in amino acid concentration as a function of development. The *C. elegans* exudates contained several compounds that cause bacterial chemotaxis, and we show that at least two species of bacteria, including the pathogenic *Pseudomonas aeruginosa*, were attracted to the exudates. This observation suggested the possibility that *C. elegans* exudates may employ a mechanism that helps protect them from pathogens. We found that *C. elegans* exudates produce a compound that inhibits quorum sensing in the reporter system based on the *LuxR* quorum sensing system (Andersen et al. 2001; Rasmussen et al. 2005a), and this compound is developmentally regulated and distinct from the compounds that cause bacterial chemotaxis. These results suggest a rich and complex set of chemical interactions between *C. elegans* and its environment.

Methods and Materials

Sample Preparation C. elegans exudates were collected at defined life stages (L1, L2, L3, L4, young adult and adult). Synchronized *C. elegans* (N2 Bristol) with a worm density of ~10,000 worms/ml were co-cultured with *Escherichia coli* (strain HB101) at 22ºC at 250 rpm on S-complete medium. The worms were harvested and separated from the medium and *Escherichia coli* by centrifugation or gravity, followed by rinsing three times over a nylon filter. They were then allowed to digest bacteria in their gut for 30 min in M9 buffer, rinsed three times in water, and incubated in water for 1 hr at density of ~30,000 worms/ml, as previously described (Jaffe et al. 1989; Johnstone 1999; Stiernagle 1999; Srinivasan et al. 2008). We recently were able to isolate and identify the *C. elegans* mating pheromone by using a similar preparation with the same relatively high worm density (Srinivasan et al. 2008). The primary purpose of the high density of worms is to collect enough material for efficient analytical characterization. All of the quantitative results shown below are normalized to the material produced by one worm in 1 h (defined as 1 worm equivalent: WE). Therefore, the density of the worm culture will not affect the outcome unless the exudates are themselves dependent on worm density. To rule out the possibility that the chemicals might be coming from dead worms, they were checked under the light microscope to verify whether they were alive before and after they were placed in water. As a negative control, we also made a similar preparation with only *E. coli* HB101 without worms. It should be noted that we are unable to distinguish whether the exudates were from regulated secretions or from defecation. The bacterial controls without worms allow us to rule out bacteria as the direct source of the exudates. For almost all experiments described below, we used the same replicate sample preps, three at each developmental stage. The exception was for the data shown in Fig. 4 in which we needed to produce additional replicates for C18 fractionation and bioassays.

NMR Data Collection from C. elegans Exudates For each life stage, *C. elegans* exudates from two independent experiments were lyophilized, and each sample was dissolved separately in 25 µl deuterium oxide (D_2O). A total volume of 12 µl containing ~54,000 worm equivalents (WE) and 0.25 mM 3-(trimethylsilyl)propionic acid-d4, a proton chemical shift reference standard (TSP=0.0 ppm), was transferred to a 1×100 mm capillary NMR tube (Norell, Inc., NJ, USA). NMR spectra of worm exudates were collected at 600 MHz on a Bruker Avance II 600 console in a 14.1 T magnet, using a 1-mm triple resonance high temperature superconducting (HTS) cryogenic probe (Brey et al. 2006). The NMR data were collected with a sample temperature of 300 ºK and spectral width of 7,211.53 Hz. The carrier frequency of ^1H was centered on residual water, which was eliminated by presaturation or by gradient methods. Two-dimensional total correlation spectroscopy (TOCSY) data were collected by using the DIPSI-2 mixing sequence (Shaka et al. 1988) with 60 or 90 ms mixing time, 2,048 complex data points along the direct and 1,024 or 512 complex data points along the indirect dimension, respectively. TOCSY data were processed with NMRPipe (Delaglio et al. 1995) by eliminating residual water by deconvolution, followed by apodization using a cosine squared function, zero-filled two times, Fourier transformed, and baseline corrected along both the dimensions. Data were analyzed and assigned with NMRview (Johnson 2004). To manually identify known metabolites by NMR, TOCSY spectra were overlaid with spectra of standard compounds downloaded from the BMRB metabolomics database (Markley et al. 2007). To confirm the different compounds identified in worm exudates, the samples were spiked with 1 to 2 mM of the respective authentic compound in D_2O, followed by the acquisition of both 1D and 2D NMR spectra.

Semi-automated Compound Identification by NMR Chemical constituents were identified using the webserver-based COLMAR protocol (Robinette et al. 2008), which provides processing and analysis of 2D NMR spectra of metabolic mixtures (http://spinportal.magnet.fsu.edu). The 2D TOCSY data were processed by using a covariance algorithm (Brüschweiler and Zhang 2004; Trbovic et al. 2004) that yields spectra with equally high resolution along both frequency dimensions. The covariance TOCSY spectra were then deconvoluted by COLMAR DemixC (Zhang

and Brüschweiler 2007; Zhang et al. 2007), which extracts 1D spectral traces that represent individual compounds by identifying spin systems with minimal likelihood of overlaps between different compounds in the covariance TOCSY spectra of the intact mixtures. In the final step, chemical shifts from individual spin systems derived from the DemixC traces were screened against the BMRB metabolomics spectral database (Seavey et al. 1991) using our COLMAR query webserver (Robinette et al. 2008; Snyder et al. 2008), which outputs a ranked list of the highest scoring compounds.

Gas Chromatography and Mass Spectrometry (GC-MS) Analysis To increase the number of identified compounds and to verify automated compound identification by NMR, we used GC-MS. We lyophilized 1 ml of young adult exudates (30,000 WE/ml) and *E. coli* (HB101) controls from three independent experiments, and chemically derivatized the lyophilized material using a two-step approach (Fiehn et al. 2000; Roessner et al. 2000; Wagner et al. 2003). Forty µl of 20 mg/ml methoxyamine hydrochloride in pyridine containing ribitol as an internal standard were directly added to the dried samples and vigorously mixed at 30°C for 1.5 h. Subsequently, 80 µl of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) containing a mixture of C_{12}, C_{15}, C_{19}, C_{22}, C_{28}, C_{32}, C_{36} n-alkanes were added and incubated at 37°C for 30 min. Derivatized samples were analyzed immediately by a TRACE DSQ GC-MS system (Thermo Finnigan Corp. Austin, TX, USA). Chromatograms were analyzed with the software AMDIS (version 2.64) in retention time index (RI) calibration mode. Metabolite identification was achieved by searching a user-library from the Max Planck Institute of Molecular Plant Physiology, Golm, Germany (Dr. Joachim Kopka, personal communication) that contains both retention time index and electron impact (EI) mass spectrum for each target component. Compounds were considered identified if their matching factor was greater than 800 on a scale of 0–1,000 and RI deviation was less than 3.0. Quantitative information for each component was extracted from the chromatogram by using the AMDIS software and a customized MACRO (http://www.lssc.nasa.gov/als/chemistry, then view features→metabolomics tool) and subsequently normalized to ribitol's response.

Quantification of Amino Acids by High Performance Liquid Chromatography (HPLC) To quantify the concentrations of amino acids present in worm exudates, an HPLC fluorescent detection methodology (Cohen and Michaud 1993) was employed. Three independent replicates of 3 ml of exudates at each developmental stage were lyophilized and resuspended in 150 µl of water. To make the amino acids fluorescent, samples were derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC, AccQ•Fluor™ Reagent Kit, Waters, Milford, MA, USA) following the manufacturer's protocol with minor modifications as indicated below. Reconstituted exudates (2.5 µl) were mixed with 17.5 µl of AccQ•Fluor borate buffer followed by the addition of 5 µl of AccQ•Fluor Reagent. The resulting mixture was incubated at 55°C for 10 min in a heating block. Prior to HPLC separation, the derivatized samples were diluted to 50 µl with water and were filtered through a 0.22-µm PVDF membrane.

The resulting fluorescent derivatives were separated on a SunFire C_{18} column (4.6×150 mm, 3.5 µm) or a Nova-Pak C_{18} column (3.9×150 mm, 4.0 µm) using a Waters Alliance 2695 separation module and were measured by a Waters 2475 fluorescence detector using an excitation wavelength of 250 nm and an emission wavelength of 395 nm. Two separation procedures under gradient conditions (Supplementary Tables S2 and S3) were used to ensure accurate identification and quantification of amino acids. Solvent composition consisted of A, water; B, acetonitrile; C1, 140 mM sodium acetate and 17 mM triethylamine, pH 5.05; C2, 100 mM sodium acetate and 5.6 mM triethylamine, pH 5.7; D, 100 mM sodium acetate and 5.6 mM triethylamine, pH 6.8. The volume of sample injected was 10 µl. The AQC-amino acid derivatives were identified and quantified by comparison with standards.

Liquid Chromatography and Mass Spectrometry (LC-MS) Analysis To search for specific known metabolites, we utilized LC-MS. One ml (30,000 WE) of worm exudates from defined developmental life stages was lyophilized and then dissolved in 50 µl of methanol. Ten µl were analyzed using a Thermo Finnigan LCQ Deca XP Max with electrospray ionization in positive and negative ion modes. Additionally, 1 nmol of commercially available patulin (MP Biomedicals, OH, USA) and penicillic acid (Acros organic, NJ, USA) were analyzed under the same conditions as above to establish retention times and diagnostic ions for screening worm exudates for these compounds.

Principal Component Analysis (PCA) We used PCA to cluster exudates from different larval stages using measurements of 14 amino acids that were quantified by HPLC. The concentrations of amino acids in worm exudates varied greatly, both among different amino acids and among the different *C. elegans* life stages for a given amino acid. For example, the concentration of Met ranges from about 0 to 0.19 pmol/WE, while Ala ranges between about 0 and 6 pmol/WE. When data are not properly scaled, the performance of PCA is dominated by high concentration

metabolites, because they constitute most of the variance. To give each metabolite an equal weight in the analysis, we scaled the data by first subtracting the mean from each metabolite and then dividing by root-mean-square. The scaled metabolite measurements have mean 0 and variance 1. PCA analysis (Johnson and Wichern 2001) was performed on the scaled data using R software (http://www.r-project.org/).

Chemotaxis Assay A slightly modified agarose plug method (Yu and Alam 1997) was used to test for bacterial chemotaxis activity of worm exudates. Briefly, bacteria grown in LB medium overnight were used as a starter culture and inoculated again in the fresh LB at a dilution of 1:50. Rifampicin (20 μg/ml) was added to *P. aeruginosa* (PAO1), because this strain is resistant to that antibiotic. No antibiotics were added to *Pseudomonas putida* (KT2440) or *E. coli* (HB101) cultures. This culture was placed in a shaker incubator until reaching an OD_{600} of 0.4–0.6, the cell density corresponding to the maximum proportion of motile cells. The cells were collected by centrifugation, washed twice with chemotaxis buffer (10 mM potassium phosphate, pH 7.0, 0.1 mM EDTA, 1 mM $MgSO_4$) and resuspended to an OD_{600} of 0.1 for the chemotaxis assay. A 10 μl drop of 2% agarose solution made with chemotaxis buffer and 10 μl of worm exudate (~300 WE) were mixed and placed on the center of the acetone cleaned microscopic slide, framed with two plastic strips (16 mm apart) and covered with a cover slip to create a chemotaxis chamber. The slide was allowed to stand for 1 min to solidify the agarose plug. Once this was achieved, 120 μl of bacterial suspension was added to the chamber surrounding the agarose plug and incubated at room temperature for 15–30 min. Following incubation, the distribution of cells immediately adjacent to the agarose plug was observed under a phase-contrast microscope and recorded with a charge-coupled device camera. Positive and negative controls were with agarose plugs containing just LB medium and chemotaxis buffer, respectively. For each trial, at least three agarose plugs were used for each developmental stage of *C. elegans* exudates. These experiments were conducted in triplicate with three independent worm exudate preparations.

Antimicrobial Assay A modified agar diffusion method (Bauer et al. 1966) was used to test for antimicrobial activity of worm exudates against the opportunistic pathogen *P. aeruginosa* (PAO1). Luria-Bertani (LB) (Bertani 1951) agar plates were prepared and spread homogenously with 100 μl of an overnight grown culture of *P. aeruginosa* (PAO1) at a density of 0.02 (10^7 cfu/ml) suspended in 10 mM $MgSO_4$ buffer. The plates were dried for 5 min under a sterile hood. Thereafter, sterile Whatman filter paper discs, 7 mm diam, were placed on the surface of seeded agar plates and loaded with 50 μl and 100 μl of filter-sterilized exudates (~1,500 and 3,000 WE) and air dried. Sterile filter paper discs, loaded with 100 μl of sterile distilled water, were used as a negative control. After the plates were incubated at 37°C for 16 h, they were examined for the presence of zones of bacterial growth inhibition. These experiments were conducted in triplicate.

Quorum Sensing (QS) Inhibition Assays We used both a QSIS1 reporter system (Rasmussen et al. 2005a) and a green fluorescent protein (GFP)-based N-acyl homoserine-lactone (AHL) sensor system (Andersen et al. 2001) to test for the QS inhibition (QSI) activity of worm exudates. Both reporter constructs are based on the LuxR system of *Vibrio fischeri* and are plasmids expressed in *E. coli* for efficient screening of QSI activity. Both assays were conducted in the presence of activating AHLs. In the QSIS1 system, a toxic gene is expressed unless an inhibitor is added, so QSI activity is required for *E. coli* growth. In the GFP-based system, GFP is expressed with AHLs, and the amount of GFP expression and thus fluorescence is reduced with added QSI activity. The medium used for this assay was ABT minimal medium supplemented with 0.5% glucose and 0.5% Casamino acids (AB medium containing 2.5 mg of thiamine/l) (Clark and Maaole 1967). Ampicillin (100 μg/ml) was added to prevent contamination of the *E. coli* assay systems. A total of 50 μl of exudate (~1,500 WE) for QSIS1 was tested. Additionally, 50 μl of chamomile extracts and sterile water served as positive and negative controls, respectively, for QSIS1.

For the *gfp*-based AHL sensor system, we used a microtiter dish and tested the QSI activity of worm exudates in the presence of 100 nM *N*-3-oxohexanoyl-L-homoserine lactone. Each well contained 100 μl of test samples, 100 μl of diluted bacterial culture (*E. coli* MT102 harboring pJBA132) in ABT medium, and 100 nM *N*-3-oxohexanoyl-L-homoserine lactone. The final bacterial density (OD_{450}) was adjusted to 0.05. GFP fluorescence was monitored at an excitation wavelength of 475 nm, and emission detection at 515 nm along with bacterial density at OD_{450} every 20 min for 7 h at 30°C using a Synergy 4 microplate reader (BioTek, Winooski, VT, USA). Sterile water and 12.5 μM 4-nitropyridine N-oxide (4-NPO) (Sigma-Aldrich) were negative and positive controls, respectively. The test samples included prefractionated young adult *C. elegans* exudates (~3,000 WE), exudate fractions that were separated by C18 solid phase extraction into flow through, 50% methanol (MeOH), and 90% MeOH fractions (Srinivasan et al. 2008), and a reconstituted fraction in which the flow through, 50 and 90% MeOH fractions were recombined.

QS-dependent Virulence Factor Assays in P. aeruginosa We also used three assays—pyocyanin, elastase, and protease—to test for QSI activity of worm exudates in *P. aeruginosa*.

The details of these assay methods and results are provided in the Supplementary Material.

Results

Chemical Composition of C. elegans Exudates We used three techniques—NMR spectroscopy, HPLC, and GC-MS—to identify the major compounds in worm exudates. These complementary techniques allowed us to identify 36 low molecular weight compounds including sugars, amino acids, and other organic acids (Table 1). Qualitative changes in exudates during *C. elegans* development can be seen in the 1D ^{1}H NMR spectra in Fig. 1. These spectra show that worm exudates contain a relatively complex mixture of compounds, some of which are constant, and some variable in concentration throughout development. The most striking changes were in methyl resonances with chemical shifts around 1 ppm (Fig. 1). These signals were strong from L2 to young adult, but significantly diminished in adult animals. Resonances with chemical shifts around 8.3 ppm, presumably from aromatic compounds, were present in adult exudates but not in those from other life stages.

Initial compound identification was achieved by overlaying TOCSY spectra of common metabolites downloaded from the BMRB metabolomics database. We also used COLMAR DemixC, a recently developed semi-automated method to analyze NMR spectra of complex mixtures (Zhang and Brüschweiler 2007; Zhang et al. 2007). About 20% of the compounds could be identified using COLMAR, including sugars (glucose and trehalose), lactic acid, and amino acids (Val, Lys, Leu, Ile, Glu) (Supplementary Fig. S2 and Table S1). All NMR identifications were verified using NMR by spiking the exudates with known pure compounds. Some additional compounds were identified by manual analysis of NMR datasets and spiking experiments with known compounds.

GC-MS profiling (Fiehn et al. 2000; Roessner et al. 2000; Wagner et al. 2003) identified 29 compounds in young adult exudates, including the 8 identified by COLMAR. These water-soluble *C. elegans* exudates include several sugars, organic acids, and amino acids (Table 1). The NMR and GC-MS analyses both demonstrate that several free amino acids are in *C. elegans* exudates. As noted above, the NMR spectra also showed that some amino acid concentrations—most obviously the methyl resonances of Val, Leu, and Ile—significantly change with development (Fig. 1). We therefore quantified amino acid concentrations by HPLC. Principal component analysis (PCA) was performed on the amino acid profile data to assess overall experimental quality and variation between each developmental stage. PCA revealed that the three highest-ranking components accounted for 90.1% of the total variance within the dataset. The first two components (81% of the total variance) were enough to distinguish the individual life stages (Fig. 2a), thus demonstrating that development is a significant factor in the variation observed in *C. elegans* exudates. Furthermore, the variance from experiment to experiment was small in

Table 1 Chemical composition of *Caenorhabditis elegans* young adult exudates[a]

Sugars	Amino acids (continued)	Other compounds
Fructose[d]	Glycine[d]	2-Ketoglutaric acid[d]
Glucose[d,c]	L-Alanine[b,e,d]	Allontoin[d]
Glucose-6-P[d]	L-Glutamic acid[b,e,d]	Anthranilic acid[d]
Trehalose[b,d]	L-Isoleucine[b,e,d]	Betaine[b]
	L-Leucine[b,e,d]	Citric acid[d]
	L-Lysine[b,e,d]	Erythronic acid[d]
Amino acids	L-Phenylalanine[b,e,d]	Fumaric acid[d]
2-Aminoadipic acid[b,e,d]	L-Serine[d]	Glutaric acid[d]
Beta-Alanine[b,e,d]	L-Threonine[e,d]	Lactic acid[b,d]
N-Acetylglutamic acid[e]	L-Tyrosine[e,d]	Malic acid[d]
Ornithine[c,e]	L-Valine[b,e,d]	myo-Inositol[d]
Proline[e]	Aspartate[e]	Threonic acid[d]
Methionine[e]	Arginine[e]	Urea[d,e]

[a] Metabolites were identified by combined approaches of NMR, GC-MS, and HPLC. [b] Identified and verified by NMR, [c] Identified but not verified by NMR, [d] identified by GC-MS, [e] identified by HPLC. Three independent experiments were done starting from worm growth for GC-MS, HPLC and only two of those used for NMR. We also analyzed four additional young adult and adult samples by HPLC. As a control, *E. coli* (HB101) was treated the same way as the worms to determine any contamination from food source. Identification of glucose-6-P is with 80% confidence.

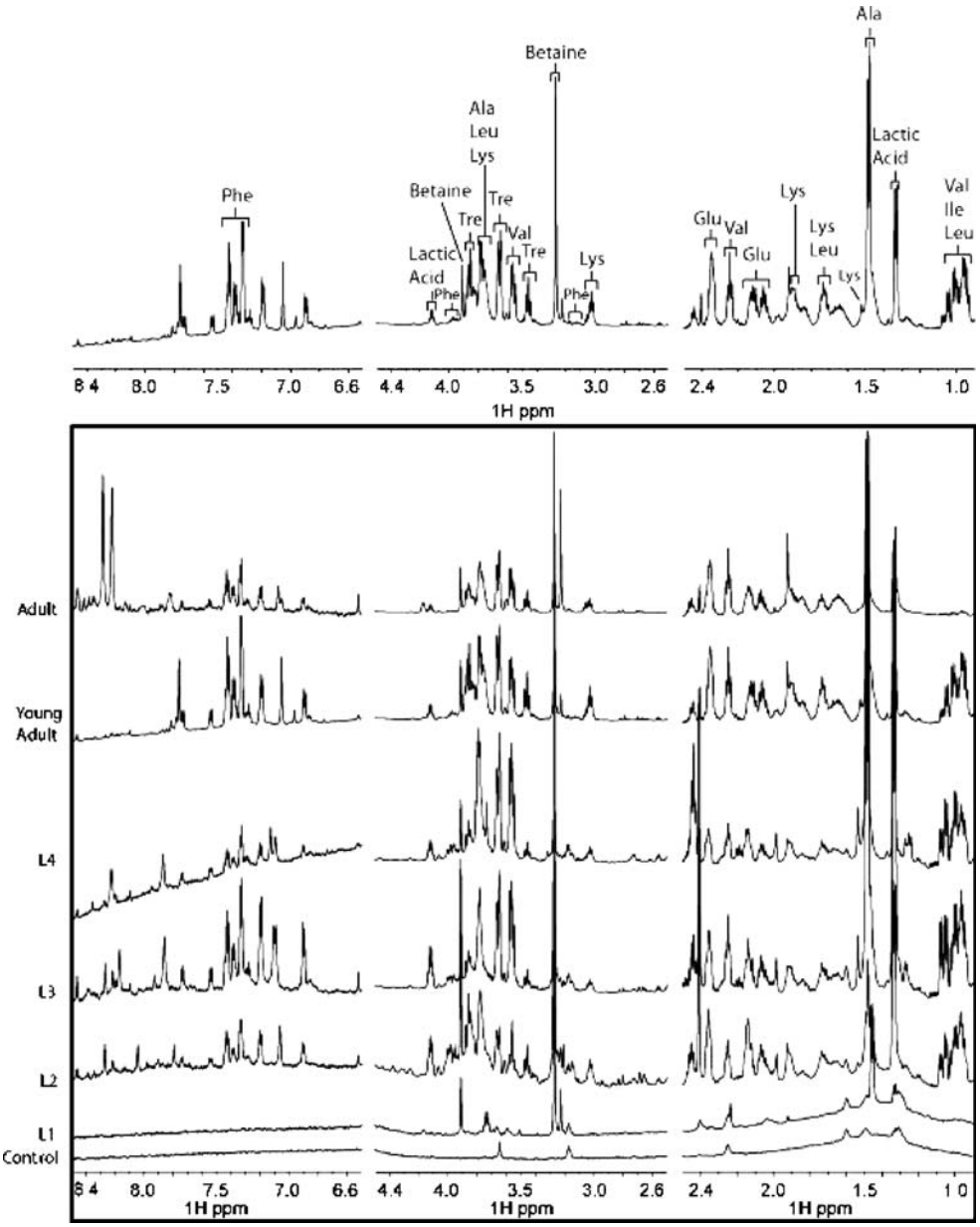

Fig. 1 NMR spectra of *Caenorhabditis elegans* exudates as a function of development. One-dimensional ^{1}H NMR spectra containing 54,000 WE from different *C. elegans* developmental stages were collected from three independent preparations of each developmental stage. The data shown are from one preparation and are representative of the others. Three regions of the spectra are shown, demonstrating a large amount of variation in chemical composition as a function of development. The annotations at the *top* are on the young adult exudates. The compounds were identified using either the semi-automated COLMAR analysis or manual analysis and spiking experiments, as described in the text

L1 and L2, but increased as the development progressed. This may be due in part to small differences in initial synchronization of worms that propagate with time.

The concentration of every amino acid in *C. elegans* exudates changed with development (Fig. 2b). Of all the developmental stages, L1 contained the fewest amino acids, with only 7 (Ala, Glu, Asp, Ile, Leu, Lys, Phe) detected and present at low concentrations. Every amino acid increased in concentration from L1 to L2 and from L2 to L3. For most developmental stages, Ala and Glu were the most abundant amino acids, with Ala at an approximately 10-fold average higher concentration than most of the others. Several amino acids (Ile, Leu, Thr, Pro, Phe, Met, Tyr, and Ala) have a profile that is highest at the L3 and young adult stages with a slight decrease at L4. With the exception of Asp, Lys, and ornithine (Orn), all amino acid concentrations decreased from young adult to adult stages. The concentration of Orn steadily increased through adult (Fig 2b).

To rule out the possibility that the identified molecules originated from the bacterial food source, we repeated the HPLC and GC-MS experiments on a control preparation that contained bacteria without worms; this sample was prepared identically to the worm exudate preparations, and we did not observe any amino acids or other compounds in Table 1. Additionally, the variation in metabolite profiles among different *C. elegans* life stages also suggests that bacteria, a constant in the experiments, are not the direct source of the observed metabolites. In summary, *C. elegans* exudates contain at least 36 amino acids, sugars and organic

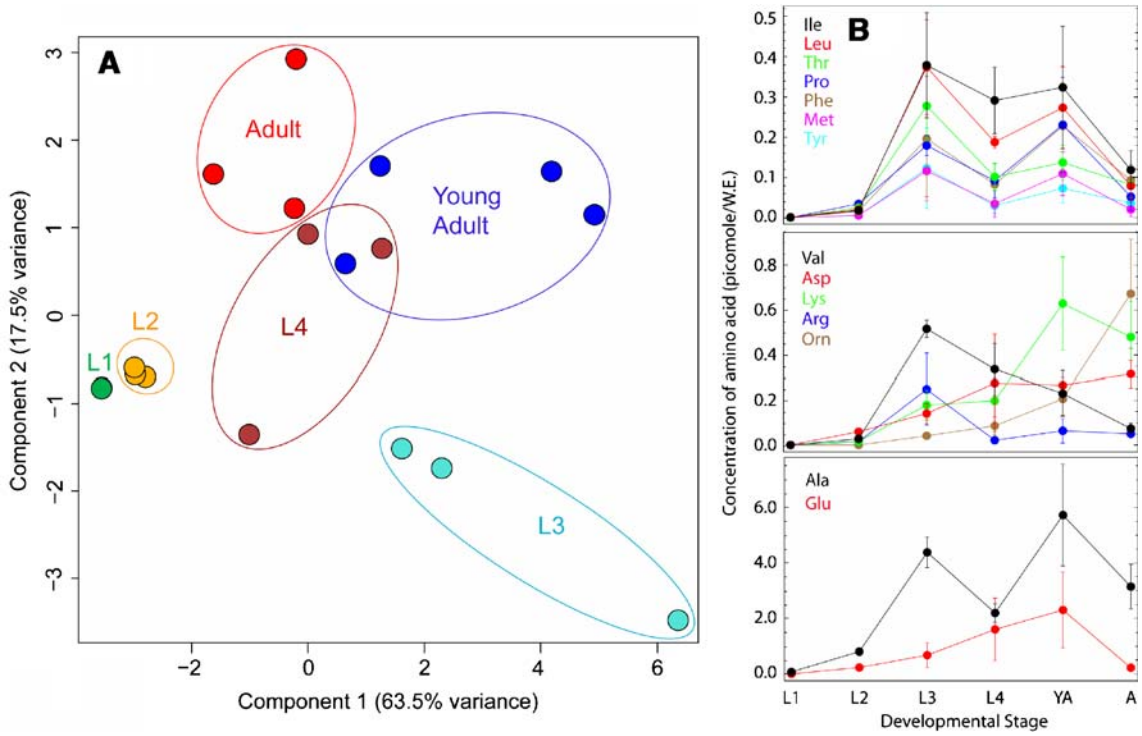

Fig. 2 Quantitative analysis of amino acids released by *C. elegans* at different developmental stages. Three independent preparations of exudates from each developmental stage were analyzed by HPLC. **a** Principal component analysis of all developmental stages. Each *circle* represents an independent experiment. The L1 data include three datasets that had very small variance among the three experiments. Components 1 and 2 account for 81% of the variation in the HPLC dataset. **b** Plots of amino acid concentrations (picomoles per worm equivalent) as a function of development. Note that the vertical concentration scale in each of the panels in **b** is different. Each point is the average and standard deviation of the three independent sample preparations and HPLC measurements. The amino acids are grouped according to overall shape of their concentration profile. The 7 in the *top* panel (Ile, Leu, Thr, Pro, Phe, Met, and Tyr) all have a peak at L3, a slight dip at L4, a slight increase at young adult (YA) and a drop at adult (A) stages. The group of four amino acids in the *middle* panel (Val, Asp, Lys, and Arg) are more variable, and the two amino acids at in the *bottom* panel (Ala and Glu) are produced in significantly higher concentrations

acids, and the concentrations of many of these compounds vary with development.

Bacteria Chemotax towards C. elegans Exudates Several species of bacteria, including *E. coli*, chemotax in response to various small molecule cues (Adler 1966; Mesibov and Adler 1972; Adler et al. 1973; Tso and Adler 1974; van der Drift et al. 1975; Hedblom and Adler 1983; Lugtenberg et al. 1999; Singh and Arora 2001; Bacilio-Jiménez et al. 2003; Kumar et al. 2007). For example, *E. coli* is attracted to Asp, Ser, Glu, Ala, Asn, Gly, Cys, Met, Thr, and trehalose (Adler et al. 1973; Hedblom and Adler 1983; Mesibov and Adler 1972) and repelled by Leu, Ile, Val, Trp, Phe, Gln, and His (Tso and Adler 1974). *C. elegans* lives in a complex soil environment with both beneficial and pathogenic microbial communities, and the chemical composition of *C. elegans* exudates suggests that the worms might attract bacteria. To test this hypothesis, exudates from young adult animals were tested qualitatively for bacterial chemotaxis by using the agarose-in-plug bridge method (Yu and Alam 1997). We tested three bacterial species: *P. aeruginosa* (PAO1), a bacterial species pathogenic to *C. elegans* (Darby et al. 1999; Aballay and Ausubel 2002), *Pseudomonas putida* (KT2440), a plant growth promoting rhizobacterium (Molina et al. 2000; Ramos-González et al. 2005), and *E. coli* (OP50), a widely used food source for *C. elegans* that is defective in motility. *P. aeruginosa* (PAO1) and *P. putida* (KT2440) both exhibited positive chemotaxis (Fig. 3a). This result is consistent with the presence of Ser, Glu, alpha-keto glutarate, succinate, citrate, and glucose in young adult exudates, as they have all been shown to attract *P. aeruginosa* (Moulton and Montie 1979). As a negative control, we tested *E. coli* (OP50), because it is widely accepted that this strain of bacteria is defective in motility (P. Sternberg, personal communication); this bacterium showed no response, consistent with our expectations (data not shown). Because beneficial and harmful bacteria were attracted to *C. elegans* exudates, we also tested the exudates for antibacterial activity against *P. aeruginosa* (PAO1). The exudates showed no antibacterial activity in our assays (data not shown). This is not surprising because previous studies documented higher bacterial activity and growth when

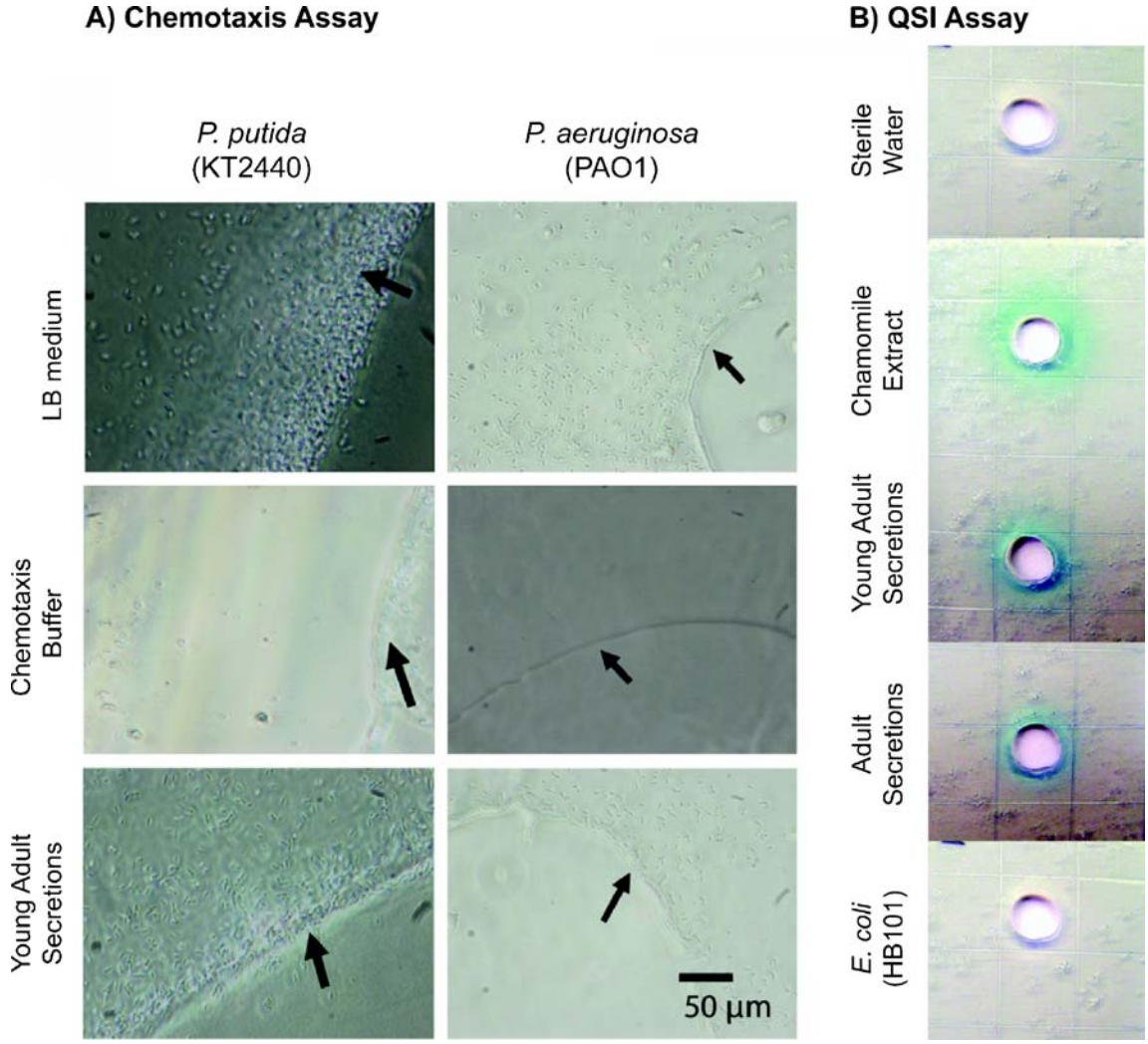

Fig. 3 Qualitative assays for bacterial chemotaxis and quorum sensing inhibition activity of *Caenorhabditis elegans* exudates. **a** Chemotaxis of *P. putida* (KT2440) and *P. aeruginosa* (PAO1). The *arrow marks* indicate the margin of the agarose plug. *Dark spots* near the margin of agarose plug are the bacterial cells. The bacterial cell density around an agarose plug that contained the exudates of interest was observed with a 40X phase-contrast objective (GX microscope) 30 min after introduction of a bacterial suspension. One plane of focus adjacent to the plug was photographed with a CCD camera on the microscope. LB media is a positive control and chemotaxis buffer is a negative control. **b** The QSIS1 reporter system (Rasmussen et al. 2005a) was employed for these qualitative assays. *Blue color* around the well indicates QSI activity. The *top* (sterile water) and *bottom* (*E. coli*) panels are negative controls, and chamomile extract is a positive control. *C. elegans* young adult and adult exudates showed positive QSI activity. For both the chemotaxis and QSI assays, three independent preparations of worm exudates were produced, and three assays were conducted on each preparation, for a total of nine assays. The figures are representative examples of one of these assays for each activity

Pseudomonas fluorescens and *Bacillus subtilis* were co-cultured with *C. elegans* (Huixin et al. 2001).

C. elegans Exudates Inhibit Bacterial Quorum Sensing (QS) We tested worm exudates for QS inhibition (QSI) activity, which would attenuate the bacterial pathogenicity, but not the growth and development (Bjarnsholt and Givskov 2007; Dong et al. 2007). All stages of *C. elegans* exudates were tested, and no activity was observed in larval stages (L1–L4). However, exudates from both young adult and adult animals showed QSI activity, as indicated by the presence of the bacterial growth around the well (Fig. 3b). Although the assay used in Fig. 3b is qualitative, the bacterial zone of growth (indicated by the blue color) is larger in exudates from young adults than adults. This suggests that *C. elegans* QSI activity is developmentally regulated.

We then tested *C. elegans* exudates for their ability to interfere with specific pathways in the production of virulence factors in *P. aeruginosa* QS. The exudates did not affect the production of elastase and protease, but induced a small amount of pyocyanin production (Supplementary Fig. S1) (Pearson et al. 1997; Fothergill et al.

2007). This small increase could be due to either an increase in release of pyocyanin, increase in biosynthesis, or both. We were unable to detect the known and commercially available QS inhibitors (patulin and penicillic acid) in *C. elegans* exudates (data not shown); these compounds have been shown to inhibit the *P. aeruginosa* QS system (Rasmussen and Givskov 2006). These results suggest that the *luxR* QSI activity of *C. elegans* exudates observed in the QSIS1 reporter system (Fig 3b) does not significantly affect the *P. aeruginosa* QS system.

Chemical Fractionation of Bacterial Chemotaxis and QSI Activities To our knowledge, the data in Fig. 3 are the first demonstration of either bacterial chemotaxis or QSI activity of *C. elegans* exudates. Based on extensive studies of bacterial chemotaxis, most of that activity could

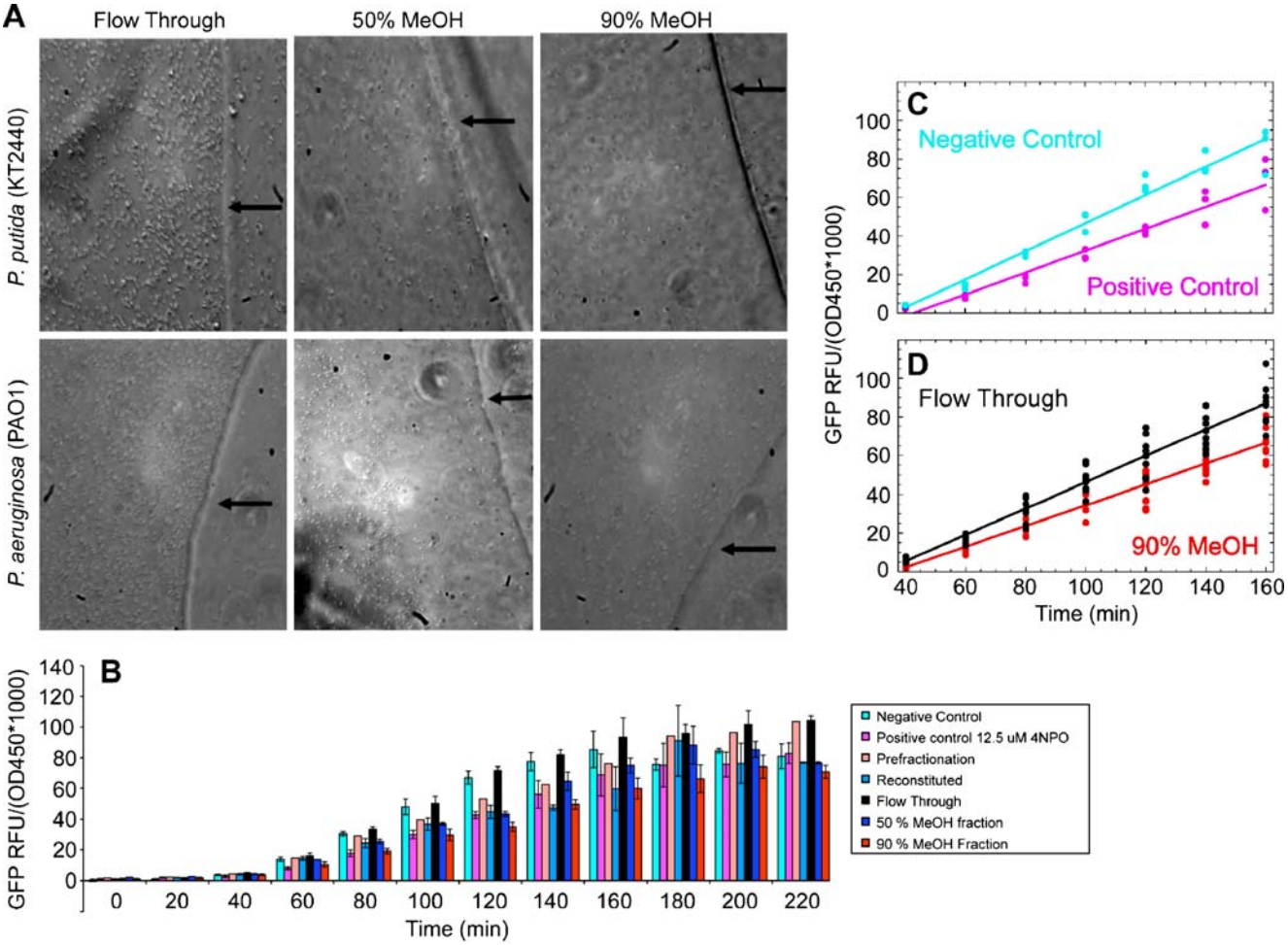

Fig. 4 Fractionation of bacterial chemotaxis and QSI activity. Young adult *Caenorhabditis elegans* exudates were separated into flow through, 50% methanol (MeOH), and 90% MeOH fractions by C18 solid phase extraction. **a** Chemotaxis of *P. aeruginosa* (PAO1) and *P. putida* (KT2440), as described in Fig. 3. The chemotaxis assay was repeated 3 times on 2 different sample preparations for a total of six replicates. Each of the assays showed the greatest bacterial chemotaxis towards the hydrophilic flow through fractions and very little chemotaxis to the most hydrophobic 90% MeOH fractions. The figures in **a** are a representative dataset. **b**, **c**, and **d** Quantitative QSI assay of young adult *C. elegans* C18 fractions (Andersen et al. 2001). **b** is a complete 220 min time course with the GFP fluorescence normalized to the bacterial optical density monitored at 450 nm. The increase in fluorescence is reduced by compounds with QSI activity, as seen by comparing the blue sterile water negative control with the pink 12.5 μM 4-nitropyridine N-oxide (4NPO) positive control. The data shown in **b** represent 3 replicate assays on 1 sample preparation. **c** and **d** show the data and linear fits from 40 to 160 min for negative and positive controls (**c**) and the flow through and 90% MeOH fractions (**d**). The negative and positive controls in **c** were from 3 replicates, and the exudate fractions in **d** were from 3 replicate measurements of 3 independent samples (total of nine measurements). The slopes, intercepts, and fitting statistics for the data shown in **c** and **d** as well as all the other conditions from **b** are in Table 2. We also compared the differences between each sample set using the model comparison technique under the linear model framework, and the statistical *P* values are shown in Table 3. The *C. elegans* flow through fraction had no QSI activity when compared with negative control ($P=0.5$), and there is no significant difference between the 90% MeOH fraction and positive control, showing that the hydrophobic fraction has QSI activity. The statistical analyses were performed using R2.8.0

Table 2 Parameters from linear regression analysis of QSI data from Fig. 4c and d[a]

Sample	Slope (STD)	Intercept (STD)	R^2
Flow through	678.7 (20.1)	−21,593.9 (3,170.1)	0.95
Negative control	729.0 (32.9)	−26,314.2 (3,540.0)	0.96
Positive control	567.7 (33.6)	−24,444.3 (3,615.1)	0.94
Prefractionation	579.3 (19.7)	−18,866.7 (2,119.2)	0.98
Reconstituded	553.0 (22.5)	−17,641.0 (3,352.4)	0.94
50% MeOH	616.4 (20.3)	−22,427.2 (3,349.5)	0.94
90% MeOH	535.2 (16.4)	−19,133.7 (3,107.7)	0.95

[a] All fits had P values <0.001.

be explained by several of the polar compounds in Table 1. In order to demonstrate that chemotaxis and QSI are caused by different chemicals, we fractionated young adult *C. elegans* exudates with a C18 solid phase extraction column (Srinivasan et al. 2008) and tested the activities of flow through as well as the 50% and 90% methanol (MeOH) fractions. The flow through contains polar compounds like many amino acids, organic acids, and sugars, which are known bacterial attractants, while the 50 and 90% MeOH fractions contain more hydrophobic compounds. Figure 4a shows that both *P. aeruginosa* (PAO1) and *P. putida* (KT2440) chemotax robustly to the flow through, less to the 50% MeOH, and very little to the 90% MeOH fractions. This is consistent with the literature on bacterial chemotaxis.

To test exudate fractions for QSI activity, we used a reporter system that could be more easily quantified and adapted to high-throughput testing than the assay used in Fig. 3b. In the assay shown in Figs. 4b, c, and d, fluorescence from the green fluorescent protein (GFP) was monitored as a function of time in a 96 well plate format. If no QSI inhibitor is present, the GFP fluorescence is maximal, and QSI inhibitors will reduce the intensity (Andersen et al. 2001). The QSI data shown in Fig. 4 were normalized to the overall cell growth monitored by the optical density at 450 nm to account for any general effects that the tested compounds have on *E. coli* cell growth. Figure 4b shows a complete time course that starts with a lag phase to about 40 min, is linear in GFP fluorescence from 40 to about 160 min, and remains stable after 160 min. Figures 4c and d are linear fits from 40 to 160 min of control (4C) and *C. elegans* flow through and 90% MeOH (4D) samples. The linear fitting parameters and correlation coefficients (R^2) and the slopes for all samples assayed in Fig. 4b are shown in Table 2, and P value comparisons of QSI activity between each sample are shown in Table 3.

The negative control (water) and *C. elegans* flow through fractions were similar (P=0.5; Table 3), demonstrating that the flow through has no QSI activity. The positive control (12.5 μM 4-nitropyridine N-oxide) and 90% MeOH fractions were also similar (P = 0.3), and the 90% MeOH fraction was distinct from both the negative control and flow through (P<0.001). These results demonstrate that the QSI activity results from a non-polar compound(s), in contrast to the polar flow through compounds that are responsible for bacterial chemotaxis. The 50% MeOH fraction has intermediate QSI activity (Tables 2 and 3). We also compared exudates before fractionation, and we reconstituted after fractionation to verify that the C18 column did not degrade or irreversibly bind to the compound responsible for the QSI activity, and these both are significantly different from the negative control (P < 0.001) (Tables 2 and 3).

Discussion

We have shown that *C. elegans* releases a variety of sugars, organic acids, and amino acids into its environment (Table 1) and that the concentrations of the amino acids are dependent upon development. Many of these compounds also are found in the exudates of other organisms including bacteria (Park et al. 2003; Zahradníčková et al. 2007), fungi (Singh and Arora 2001), and several species of

Table 3 The *Caenorhabditis elegans* flow through has no QSI activity, and the 90% MeOH fraction has similar activity to the 12.5μM 4NPO positive control

	NC	PC	Pre	Rec	50% MeOH	90% MeOH
Flow through	5.10E-01	<0.001	<0.001	<0.001	<0.001	<0.001
Negative Control (NC)		<0.001	<0.001	<0.001	<0.001	<0.001
Positive Control (PC)			<0.001	1.27E-02	<0.001	3.17E-01
Prefractionation (Pre)				5.43E-01	6.79E-01	4.56E-03
Reconstituted (Rec)					1.19E-01	3.97E-02
50% MeOH						<0.001

[a] The entries are P values, and numbers greater than 0.05 indicate that the two samples are not statistically different. The highlighted boxes show that the *Caenorhabditis elegans* flow through has no QSI activity and that the 90% MeOH fraction has similar activity to the 12.5 μM 4NPO positive control.

plants (Lugtenberg et al. 1999; Bacilio-Jiménez et al. 2003; Kamilova et al. 2006; Kumar et al. 2007). Additionally, many plant root exudates, and fungal or bacterial exudates attract bacteria (Bacilio-Jiménez et al. 2003; Kumar et al. 2007; Lugtenberg et al. 1999; Singh and Arora 2001; Park et al. 2003). Given their chemical composition, it was not surprising that *C. elegans* exudates examined in this work attracted bacteria. Previous experiments have shown that *C. elegans* enhance the growth of *Pseudomonas fluorescens* and *Bacillus subtilis* (Huixin et al. 2001). This suggests a hypothesis that *C. elegans* plays an active role in soil chemical ecology and may have evolved chemical systems for attracting and promoting growth of its own food source. Relatively little is known about the range of bacterial species that *C. elegans* eats in its native environments (Avery and Shtonda 2003; Shtonda and Avery 2006), so the hypothesis of "bacterial farming" by *C. elegans* remains to be tested. It should be noted that we investigated only water-soluble compounds that are released from the worms, and other compounds may interact with the hydrophobic cuticle or may be volatile.

If *C. elegans* non-specifically attracts bacteria, it is possible that pathogenic species have evolved to take advantage of this otherwise beneficial survival mechanism. Depending on the bacterial composition in a particular environment, one might predict that *C. elegans* would also need, therefore, to employ some sort of defense against pathogens or colonize soil patches that are devoid of pathogens. However, we were unable to detect any *C. elegans* antibacterial activity against the pathogenic bacterium *P. aeruginosa*, despite the fact that this same pathogen was attracted to *C. elegans* exudates. Since *P. aeruginosa* is resistant to many antibiotics (Stover et al. 2000), we cannot completely rule out the possibility that *C. elegans* produces antibacterial compounds, or that antibacterial compounds might be present in the intestine or on the cuticle of the nematode rather than released. Even though the data and literature are best explained by the absence of antibacterial compounds in *C. elegans* (Huixin et al. 2001), a result that is ecologically consistent with a bacterivorous nematode, this does not eliminate the possibility that *C. elegans* may have an inducible system to produce antibacterial activity.

Many pathogenic bacteria, including *P. aeruginosa*, regulate virulence through quorum sensing (Bjarnsholt and Givskov 2007; Dong et al. 2007), and *P. aeruginosa* (PAO1) is lethal to *C. elegans via* a toxin produced through the *LasR* QS system (Darby et al. 1999). Many species of bacteria, fungi, and plants employ a variety of mechanisms that inhibit bacterial QS, including enzymatic degradation of QS signaling molecules and direct inhibition (Walker et al. 2004; Bais et al. 2005; Dong et al. 2007). We showed that *C. elegans* exudates inhibited a *LuxR* QS system that was derived from *Vibrio fischeri*. However, the worm exudates did not affect common biomarkers of QS in *P. aeruginosa* (PAO1), the production of elastase and protease, and they had only a small influence on pyocyanin production (Supplementary Fig. S1). The *C. elegans* QSI activity appears developmentally regulated with maximal activity at the young adult stage. Perhaps *C. elegans* has evolved a mechanism by which it can avoid infection by certain pathogenic species of bacteria without employing antibiotics. These results are consistent with the observations that *C. elegans* exudates attract several bacterial species, as the QSI activity would provide the nematodes with a chemical defense without killing their food source.

In summary, *C. elegans* releases many common small molecules that can modulate its environment, and many of these compounds are developmentally regulated. These compounds may play important roles in the ecology of *C. elegans* in its natural habitat. Our data suggest that these molecules can provide a means for the worms to interact with bacterial food sources and potentially to defend against bacterial pathogens. It remains an open question whether the bacterial chemoattractants or quorum sensing inhibitor(s) are evolutionary adaptations or are byproducts of another process, such as metabolism. The identification of the QSI will help address this question and may open up new areas of research into the chemical ecology of nematodes.

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RAPID COMMUNICATION

Identification of Secretory Compounds from the European Callipodidan Species *Apfelbeckia insculpta*

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Abstract Defensive secretions of the callipodidan species *Apfelbeckia insculpta* contain a *p*-cresol as the main component and phenol in traces. This is the first identification of these compounds in a European callipodidan species. The repugnatory glands of *A. insculpta* are of the spirobolid type and consist of a spherical reservoir, a simple duct, and a valvular cuticular infolding that opens onto the lateral surface of the millipede via a pore.

Keywords Defensive secretion · Callipodida · *Apfelbeckia insculpta* · *p*-cresol · Phenol · Diplopoda

Introduction

Defense glands are present in all millipedes except for Penicillata, Sphaerotheriida, Chordeumatida, and Glomeridesmida (Hopkin and Read 1992; Shear et al. 2007). Julida, Spirostreptida, and Spirobolida were called by Eisner et al. (1978) "quinone millipedes" due to the universal presence of quinones in their defensive fluids. The orders Spirobolida and Spirostreptida emit secretions that contain mainly 1,4-benzoquinone and hydroquinone derivatives (Omura et al. 2002). The order Polydesmida was called by Eisner et al. (1978) a "cyanogenic order" because it includes millipedes that produce hydrogen cyanide, benzaldehyde, and mandelonitrile (Omura et al. 2002). Mandelonitrile is considered to be a biogenetic precursor of benzaldehyde and hydrogen cyanide. Some polydesmidan species also emit phenol derivatives (Blum et al. 1973; Duffey et al. 1977). Secretions from representatives of the orders Glomerida and Polyzoniida contain characteristic alkaloids (Omura et al. 2002). There are only a few published data concerning defensive secretions in members of the order Callipodida (Eisner et al. 1963; Shear et al. 2007).

The order Callipodida Bollman 1893 contains a wide variety of disjunct forms probably with relict status. The given order comprises three suborders (Sinocallipodidea Shear 2000; Callipodidea Pocock 1894; and Schizopetalidea Hoffman 1973), seven families, 35 genera, and 133 (sub-) species (Stoev et al. 2008). According to published data, a defensive secretion has been documented only in three callipodidan species found in the Southern Appalachians in the United States—*Abacion magnum* (Loomis 1943), *Tetracion jonesi* Hoffman 1956, and *Delophon georgianum* Chamberlin 1943—belonging to the suborder Schizopetalidea and family Abacionidae Shelley 1979 (Shear et al. 2007). These three species produce only *p*-cresol as a defensive secretion (Shear et al. 2007).

There is no published report of defensive secretions in European callipodidans. For these reasons, we focused our research on the species *Apfelbeckia insculpta* (L. Koch 1867), which belongs to the genus *Apfelbeckia* Verhoeff

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1900, suborder Schizopetalidea and family Schizopetalidae Verhoeff 1909. This species is troglophilic (distributed on the Balkan Peninsula), and when disturbed emits a secretion with a strong smell of cresols. The present study represents the first attempt to clarify the chemical composition of the secretion in a European callipodidan species.

Methods and Materials

Millipedes of the species *Apfelbeckia insculpta* at subadult and adult stages were collected in March of 2008 from two caves in Western Serbia: Hadži-Prodanova Pećina Cave, village of Raščići, near Ivanjica; and Mlađenovića Megara Cave, village of Stapari, near Užice. The collected millipedes were sexed (five males, five females, and five juveniles from both localities) and separated in plastic dishes (20 cm diam×10 cm in height) with cave substrates. The millipedes were kept in the laboratory for a few days at 10°C in darkness under humid conditions created by spraying water every day.

For collection of defensive secretions, each millipede was soaked individually in methanol (1 ml) for 3 min. The methanol extract was removed by decantation and filtered through a 0.45 μm poly-tetrafluoroethylene (PTFE) filter (Agilent Technologies). To eliminate the effects of composition-altering oxidation and degradation of compounds, a portion of the extracts was subjected to GC-MS analysis immediately after preparation.

The GC-MS analyses were performed on an Agilent Technologies 5975C mass spectrometer coupled with a 7890A gas chromatograph by using an HP-5 MS capillary column (Agilent Technologies, 0.25 mm I.D.×30 m, 0.25 μm film thickness) under programmed conditions of temperatures that were varied from 60°C to 290°C at a rate of 10°C/min with an initial 2-min hold. Samples were injected using a splitless mode at 240°C and He as the carrier gas (constant flow, 1 ml/min). Mass spectra were obtained by electron ionization at 70 eV, the ion source temperature being 230°C.

Results

Gas chromatography-mass spectrometry (GC-MS) of the methanol extracts in all specimens (males and females, as well as juveniles) of *A. insculpta* from both localities showed two volatile compounds, *p*-cresol as the main component and phenol in traces. These compounds were identified by comparison of mass spectra in the NIST MS Search 2.0 computerized mass spectral library and confirmed by comparison with the retention time (t_R) and mass spectra of authentic standards.

Discussion

Apfelbeckia insculpta possesses a pair of repugnatorial glands on each of the pleurotergites. Only the first five pleurotergites and the last one (or two) are devoid of glands. The defense glands consist of a spherical sac or reservoir that contains secretory cells, a simple duct, and a valvular cuticular infolding that opens onto the lateral surface of the millipede via a pore (Fig. 1). The same type of repugnatorial gland was found in the callipodidan species *Abacion magnum* (Eisner et al. 1963) (the authors assigned the genus *Abacion* to the group of 'chordeumoid' millipedes; it belongs to the order Callipodida). This type of gland also is present in millipedes of the orders Spirobolida, Spirostreptida, Julida, Platydesmida, and Polyzoniida (Hopkin and Read 1992) and is classified as the spirobolid type. During collection, we observed that when disturbed, *A. insculpta* coils up into a tight spiral, keeping its head in the center. If further disturbed, the millipede discharges a defensive secretion. A second type of glands in millipedes (the polydesmid type) consists of a reservoir, duct, vestibule, and external opening. This type is characteristic of the order Polydesmida (Hopkin and Read 1992).

p-Cresol has been reported previously from three callipodidan species, viz., *Abacion magnum*, *Tetracion jonesi*, and *Delophon georgianum* (Abacionidae) (Shear et al. 2007). We found it in methanol extracts of a schizopetalid species, *A. insculpta*. Such a finding supports the idea (Shear et al. 2007) that from the known chemical composition of defensive secretions in these millipedes,

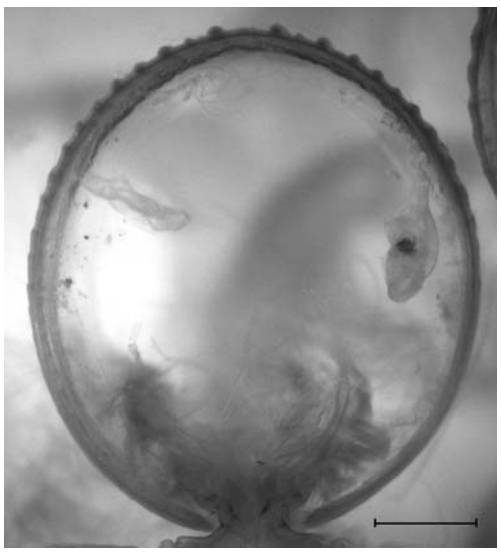

Fig. 1 Defense glands in one midbody pleurotergite in a male of *Apfelbeckia insculpta* (L. Koch 1867) from Hadži-Prodanova Pećina Cave, village of Raščići, near Ivanjica, Serbia, anterior view. Scale line=2 mm

p-cresol is probably the main characteristic secretion of all callipodidans. Apart from Callipodida, *p*-cresol is found also in millipedes of the order Polydesmida and in parajulid julidan *Oroiulus venustus* (Wood) (Eisner et al. 1978; Shear et al. 2007).

Little information is available regarding natural predators of *A. insculpta*. However, unlike almost all other millipedes, *A. insculpta* is a predator that feeds on earthworms, flies, spiders, and centipedes (Hoffman and Payne 1969). In the analyzed cave systems, the number of potential predators of adult specimens of this species is limited. Only early postembryonic stages may be a target of some spiders or carabids inhabiting the same caves from which *A. insculpta* was collected. On the other hand, cave systems represent an important shelter ensuring relatively safe reproduction (this is supported by the fact that in both cave systems studied, we found almost all postembryonic stages of *A. insculpta*).

On the basis of the aforementioned facts, we suggest that *p*-cresol in *A. insculpta* functioned as a defensive allomone. *p*-Cresol is known to be an effective repellent of ants and probably other insects (Eisner et al. 1978). It also repels lizards, jays, and grasshopper mice (Eisner et al. 1963), and may damage the potential predator. It may be important during the early postembryonic development of *A. insculpta*, as well as in adult stages if they ever leave the cave shelter. Furthermore, it is known that *p*-cresol and phenol exert antifungal activity by suppressing mycelia growth and spore germination (Roncadori et al. 1985). It is important in connection with this that in both cave-systems during collection of specimens, we registered abundant fungal populations. Other potential roles of *p*-cresol (chemical messenger or pheromone) will be the subject for further investigation.

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Sensory Imbalance as Mechanism of Orientation Disruption in the Leafminer *Phyllocnistis citrella*: Elucidation by Multivariate Geometric Designs and Response Surface Models

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Abstract Experimental designs developed to address mixtures are suited ideally to many areas of experimental biology, including pheromone blend studies, because such designs address the confounding of proportionality and concentration intrinsic to factorial and one-factor-at-a-time designs. Geometric multivariate designs coupled with response surface modeling allowed us to identify optimal blends of a two-component pheromone for attraction and trap disruption of the leafminer moth, *Phyllocnistis citrella*, a major pest in citrus growing areas around the world. Field trials confirmed that the natural 3:1 blend of (*Z,Z,E*)-7,11,13-hexadecatrienal:(*Z,Z*)-7,11-hexadecadienal was most effective as an attractant for male moths. However, the response surface generated in mating orientation trials revealed that the triene component alone was more effective than the natural blend in disrupting trap catch. Each individual component was effective at disrupting orientation in field trials, but (*Z,Z,E*)-7,11,13-hexadecatrienal was approximately 13 times more effective, at the same concentration, compared with (*Z,Z*)-7,11-hexadecadienal alone. In addition, the application of geometric design and response surface modeling to field studies provided insight into a possible mechanism of mating disruption and supported sensory imbalance as the operating mechanism for this species.

Keywords Mating disruption · Pheromone blends · Mixtures · Citrus leafminer · Non-competitive disruption · Citrus canker

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Introduction

Many insects, particularly moths, rely upon pheromonal communication for recognizing and locating potential mates (Cardé and Minks 1995). Although some species use a single compound as a sex pheromone (Novak and Roelofs 1985), most deploy a blend of compounds in species-specific ratios. The production of synthetic sex pheromones to disrupt mating in insect pest species has become a sizeable industry that contributes to a reduced reliance on insecticides in integrated crop protection strategies. Synthetic sex pheromones for mating disruption of crop pests are used on an estimated 662,000 ha of land worldwide (Witzgall et al. 2008).

At least two general models have been proposed to explain the mechanism by which treatment of crops with sex pheromones results in disruption of sexual communication and subsequent reduction in population densities. Competitive models suggest that sources of synthetic

pheromone compete with calling females. Under this scenario, disruption occurs when males follow false plumes and are, therefore, unable to locate females (Miller et al. 2006a). Non-competitive models hold that permeation of the air space with synthetic pheromone interferes with perception of the female's signal by camouflage, desensitization, and/or sensory imbalance (Miller et al. 2006a). Experimental approaches for distinguishing competitive from non-competitive disruption have been proposed based on transformations of moth catch data in traps as a function of pheromone dispenser density per unit area of crop (Miller et al. 2006a, b).

While it is generally understood that proportionalities of multi-component pheromone blends are important in insect attraction, it is not clear whether mating disruption is best achieved by using the natural pheromone blend or some other "unnatural" ratio (Minks and Cardé 1988). The development of mating disruption technology remains largely an empirical process of testing blends and concentrations (Cardé 1990).

Studies of blends, in general, and pheromone blends in particular, often confound the effects of proportionality and amount by allowing them to vary in parallel [cf. Evens and Niedz (2008) for a discussion on confounding in proportion-amount experimental designs]. Distinguishing between proportionality and concentration effects requires a special design known as a "mixture-amount" experiment (Cornell 2002), wherein proportionality and concentration are treated independently. In addition, commonly applied experimental designs such as factorial or one-factor-at-a-time approaches are either inherently confounded or fail to explore the experiment's design space adequately. For rational development of mating disruption technology, an experimental approach is required that distinguishes between proportion and amount, efficiently allocates treatments through a potentially complex multidimensional design space, and accounts for complex interactions between variables in multi-component pheromone systems.

The leafminer, *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae), is a major pest of citrus throughout the world (Heppner 1993). Larvae feed within leaves, producing serpentine mines that result in distortion and loss of photosynthetic capacity, thereby reducing yield (Peña et al. 2000). In addition to direct damage, mining by *P. citrella* increases the susceptibility of citrus leaves to infection by citrus canker, *Xanthomonas axonopodis* pv. *citri*, an important disease of citrus (Gottwald et al. 2007). Our interest was to determine optimal pheromone blends for attraction and mating disruption and to gain insight into the mechanism of disruption for control of *P. citrella* through application of appropriate experimental design. We used mixture-amount designs to quantify optimal pheromone blends and concentrations for both attraction to traps and orientation disruption in a series of field experiments conducted in Florida citrus groves. We believe the methods employed here to be of wide applicability, within this field and many other fields of biological inquiry, in which responses to proportion and amount of mixture components are measured.

Methods and Materials

Pheromone Synthesis

(*Z,Z,E*)-7,11,13-Hexadecatrienal and (*Z,Z*)-7,11-hexadecadienal (referred to also as triene and diene, respectively) were synthesized by ISCA Technologies Inc. Riverside, CA, USA, as previously described (Leal et al. 2006; Moreira et al. 2006), resulting in (*Z,Z,E*)-7,11,13-hexadecatrienal of 94% chemical purity (90% isomeric purity) and (*Z,Z*)-7,11-hexadecadienal of 86% chemical purity (72% isomeric purity).

Attraction Trial

Red, 11-mm natural rubber septa (West Pharmaceutical Services Co., Lionville, PA, USA) were loaded with hexane solutions (100 µl) of test blends of *P. citrella* sex pheromone, to yield 23 treatments consisting of varying proportions and amounts of the triene and diene (Fig. 1a). Thirteen of the treatments were replicated twice to estimate pure error. Blend proportion varied from 100% triene to 100% diene, and the amount of blend loaded onto each septum varied from 0.01 to 1.0 mg. Individual septa containing the pheromone blends were placed in traps (Pherocon VI, Trécé, Inc., Adair, OK, USA), with sticky card liners, and randomly assigned to field locations within a 12 ha mature production block of Duncan grapefruit trees, near Ft. Pierce, FL, USA, on 22 May 2008. Traps were placed at 45 m intervals within rows, with three traps per row. Rows with traps were separated (45 m) by six rows without traps. Adhesive liners were replaced and examined for the presence of *P. citrella* 24 h after deployment and weekly thereafter.

Disruption Trial

A commercial emulsified-wax matrix designed to provide slow release of semiochemicals (SPLAT^{TM}, ISCA Technologies) was formulated with varying proportions and amounts of (*Z,Z,E*)-7,11,13-hexadecatrienal and (*Z,Z*)-7,11-hexadecadienal. Proportions varied from 100% triene to 100% diene, and amount varied from 0.02 to 2.0 g/kg (0.002 to 0.2%) of formulated SPLAT (Fig. 1b) for a total of 17 proportion/amount combinations in separate plots and 3 control plots (no SPLAT). Eight of the treatments

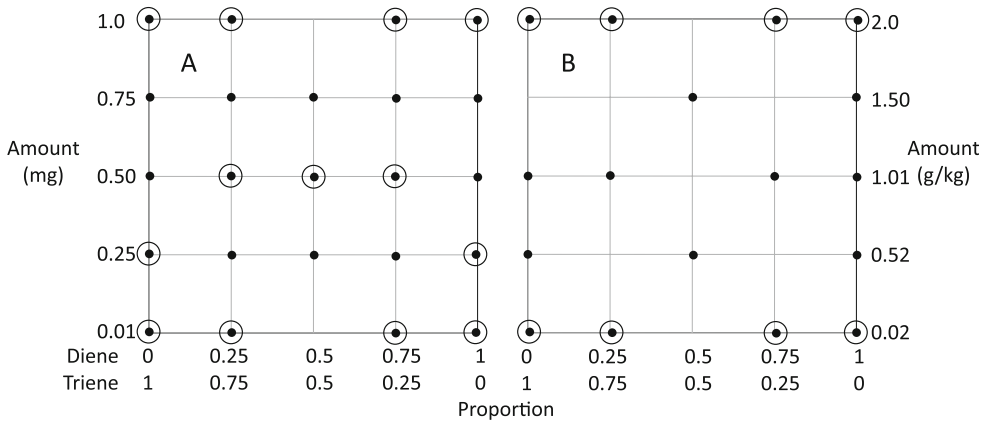

Fig. 1 A: Mixture/amount experiment design space showing 23 design points, consisting of varying ratios and amounts of (*Z,Z,E*)-7,11,13-hexadecatrienal (triene) and (*Z,Z*)-7,11-hexadecadienal (diene), to assess response of *Phyllocnistis citrella* males to pheromone-baited traps in a Florida citrus grove. The thirteen circled points were replicated twice for a total of 36 proportion-amount combinations (runs). B: Design space containing 17 design points, of varying ratios and amounts of triene and diene, to assess response of *P. citrella* males to pheromone-baited traps within 0.2 ha plots treated with a slow-release pheromone delivery system (SPLAT®) in a Florida citrus grove. The eight circled points were replicated twice for a total of 28 runs

were replicated twice to estimate pure error. SPLAT treatments and controls were randomly assigned to 0.2 ha plots of 77 trees (7 rows x 11 trees/row), equally spaced within a 24-ha mature production block of Duncan grapefruit trees, near Ft. Pierce, FL. SPLAT treatments were applied on 23 April 2008 using hand-held applicators calibrated to deliver 1 g dollops. Point source density and concentration were based on results previously reported for the 3:1 triene:diene blend (Stelinski et al. 2008). Within plots, each tree received 3 dollops of SPLAT at approximately a 1.5 m height. Perimeter trees received 4 dollops.

Trap shutdown, the disruption of male catch in traps baited with synthetic lures containing the natural blend of sex pheromone, was employed to quantify disruption. To measure trap shutdown, two Pherocon VI traps, each baited with a single rubber septum (West Pharmaceutical Services Co., Lionville, PA, USA) loaded with 130 µl of a hexane solution containing 130 µg of a 3:1 blend of (*Z,Z,E*)-7,11,13-hexadecatrienal and (*Z,Z*)-7,11-hexadecadienal, were placed in the center row of each plot equidistant from each other and the plot perimeter (trees 4 and 8 in rows of 11 trees each). Lures were deployed in traps on 23 April 2008 and replaced weekly.

Experimental Design

D-Optimality criteria were used to minimize the number of factor/component combinations necessary to provide accurate estimates of the coefficients of a quadratic x quadratic Scheffé polynomial model (Cornell 2002; Box and Draper 1971). Specifically, sufficient treatments or 'design points' (Fig. 1) were selected with Design Expert® software (v7.0.3, Stat-Ease, Inc. Minneapolis, MN, USA) such that the determinant of the $(X'X)^{-1}$ matrix was minimized; this has the net result of minimizing the volume of the confidence ellipsoid for the coefficients of the selected model (Piepel 1982). Several design points were added to estimate the lack of fit (LOF) between the response surface models and design points not used to generate the model fits (Weisberg 1985). A number of treatments were duplicated in order to: 1) attain sufficient degrees of freedom (df) to estimate pure error across the design space; 2) provide estimates of block effects; and 3) reduce the potential effect (s) of high leverage points. In all, there were 36 runs (proportion-amount treatments) for the attraction study and 25 runs for the disorientation study.

Statistical Analyses

A detailed description of the statistical methods used to analyze the data can be found in Lapointe et al. (2008). Briefly, all possible models from the mean to cubic polynomial were calculated with Design Expert® v.7.0.3. Initial model selection was based on a battery of adequacy tests (Anderson and Whitcomb 2005). Normality and constant variance were determined graphically; a Box-Cox plot was used to choose the correct transformations (Box and Cox 1964). Overly influential data points were identified with DFFITS and DFBETAS plots (Belsley et al. 1980). Adequate model precision was determined by comparing the range of predicted values at design points ($\hat{y}$) to the average variance (V-bar) of the prediction (Anderson and Whitcomb 2005). Potential outlier points were checked with externally studentized "outlier-t" (Weisberg 1985; Myers 1990) and Cook's Distance (Cook and Weisberg

1982) graphical plots. R^2, adjusted-R^2 (R_{adj}^2), and predicted-R^2 (R_{pred}^2), were estimated for each selected model [cf. Myers and Montgomery 2002)].

Test of High Diene SPLAT Disruptant

An estimated relative cost of synthesis of the diene and triene provided by ISCA Technologies was included in a model of results from the disruption trial. The resulting plot suggested that diene alone, if deployed at a much higher concentration, might function as a disruptant of trap catch as well as the triene alone. Therefore, a separate field trial was conducted in October 2008 to test the efficacy of a high concentration of the diene component for disrupting trap catch compared with the triene-only treatment identified as optimal in the disruption trial. Plots (0.2 ha) in a mature citrus grove were as described above for the disruption trial. SPLAT formulations consisted of 2.0 and 20.0 g/kg of diene, and 1.5 g/kg of triene. Four replicates of each of the three SPLAT treatments were applied on 3 October 2008. Four replicates with no SPLAT were included as controls. Two traps baited with the natural 3:1 blend were deployed in the center of the plots to assess trap shutdown as described above. Counts of trap liners were conducted every 3 to 4 d for a period of 26 d. Linear regression was used to describe the percent disruption of trap catch obtained in the treated plots compared with the control plots.

Results

Attraction Trial

There were no significant models that included amount of (*Z*,*Z*,*E*)-7,11,13-hexadecatrienal and (*Z*,*Z*)-7,11-hexadecadienal on capture of male moths (*P*-values ranged from 0.07 to 0.91). Therefore, amount effects were averaged across this dimension, which resulted in a highly significant (*P*-value <0.001) cubic x mean polynomial response surface model (RSM) for the number of male moths captured in pheromone-baited traps (Table 1; Fig. 2). Data were transformed (1/sqrt) based on the best lambda value from the Box-Cox plot [see Lapointe et al. (2008) for complete statistical methods]. The three R^2 statistics (R^2, R_{adj}^2 and R_{pred}^2) were ≥0.92. The greatest trap catch was obtained at a 3:1 triene:diene pheromone blend. "Pure" blends consisting of various amounts of the diene component alone were not attractive. While pure blends of triene showed some attraction (6% of the maximum 3:1 blend), trap catch decreased markedly as the lure blend diverged from the 3:1 ratio (Fig. 2) in either direction of the blend ratios.

Disruption Trial

The disruption trial produced a highly significant (*P*-value <0.001) linear x linear polynomial RSM (Table 1; Fig. 3) for trap shutdown, calculated as trap catch expressed as a percent of the control (no SPLAT pheromone applied). There was a highly significant interaction between triene proportion and amount, and also a significant interaction between diene proportion and amount (Table 1). No significant blending effect between diene and triene was observed; the highest level of trap shutdown occurred with pure triene (Fig. 3).

Test of High Diene SPLAT Disruptant

The high concentration of diene in SPLAT contained 40x the amount of diene in the highest concentration of the natural 3:1 blend tested in the disruption trial. Percent trap shutdown obtained in plots treated with the high concentration of diene (20 g/kg) and triene (1.5 g/kg) was similar (Fig. 4). The high diene treatment resulted in 89% reduction of moth catch in traps relative to untreated control plots over the 26 d duration of the trial. The 0.15% triene treatment resulted in an 83% reduction over the same period.

Discussion

Experimental designs developed to study mixtures (Cornell 2002) are ideally suited for pheromone blend studies. An inherent difficulty in quantifying main effects and interactions of mixture components is the confounding effects of proportionality and concentration, intrinsic to traditional factorial experimental designs. Confounding occurs when two or more quantities are varied together in a manner that makes it mathematically impossible to identify their unique effects (Evens and Niedz 2008). To overcome this, blends can be conceptualized as mixtures in which the sum of the component proportions is one. Because of the proportionality constraint, the resulting experimental design space has *n*-1 dimensions. The addition of one additional dimension to account for amount completes the design space. For example, the two-component pheromone system of *P. citrella* requires only a two-dimensional design space, one dimension for the mixture (i.e., ratio) of the two components, and one dimension for amount. Current computer hardware and software make it possible to design, execute, and analyze efficient experiments that systematically sample multidimensional experimental design space and generate mathematical equations that describe response variables across the entire design space with a minimum number of runs. Such an approach is more cost effective

Table 1 ANOVA and model diagnostic data for the cubic x mean and linear x linear Scheffé polynomial models developed for attraction and disruption responses of *Phyllocnistis citrella* in field trials, respectively

Source	Attraction		Disruption	
	F-value	*P*-value[a]	*F*-value	*P*-value[a]
Model	158.4	**< 0.001**	23.8	**< 0.001**
Linear Mixture	136.0	**< 0.001**	18.1	**<0.001**
Diene[b] x Amount	n/a		7.0	**0.016**
Triene[b] x Amount	n/a		36.4	**< 0.001**
Diene x Triene	177.4	**< 0.001**	n/a	
D x T (D - T)	161.7	**< 0.001**	n/a	
Lack of Fit		0.226	3.9	**0.031**
Std. Dev.	0.05		0.19	
Mean	0.41		0.71	
C.V. %	212.86		6.73	
R^2	0.94		0.78	
R^2adj	0.93		0.75	
R^2pred	0.92		0.69	
Polynomial Coefficient Estimate				
	1/Sqrt =		% of Control =	
Diene	0.60		0.92	
Triene	0.47		0.48	
Diene x Amount	-n/a		0.19	
Triene x Amount	-n/a		0.45	
Diene x Triene	−1.15		n/a	
D x T (D - T)	1.99		n/a	

[a] Significant *P*-values (α=0.05) are indicated in bold

[b] diene = (*Z,Z*)-7,11-hexadecadienal, triene = (*Z,Z,E*)-7,11,13-hexadecatrienal

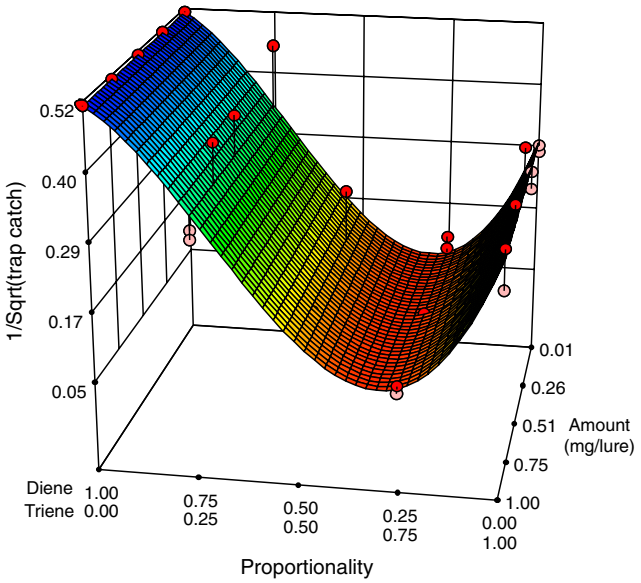

Fig. 2 Three-dimensional plot of the inverse of the square root of number of male *Phyllocnistis citrella* moths caught in pheromone-baited traps at two weeks after deployment, as a function of pheromone component proportionality and total pheromone amount. Greatest trap catch was achieved with the natural 3:1 ratio of (*Z,Z,E*)-7,11,13-hexadecatrienal (triene): (*Z,Z*)-7,11-hexadecadienal (diene)

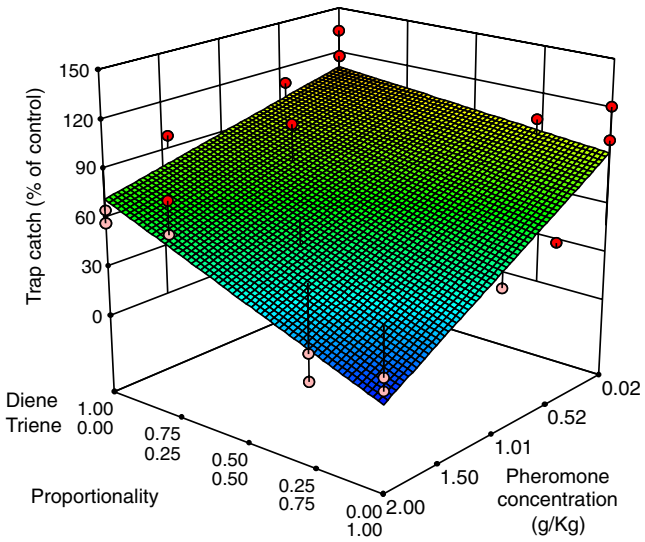

Fig. 3 Three-dimensional plot of trap catch within disruptant-treated plots expressed as percent of control trap catch at two weeks after deployment of SPLAT treatments. The lowest number of male *Phyllocnistis citrella* were captured in traps [baited with a 3:1 (*Z,Z,E*)-7,11,13-hexadecatrienal (triene): (*Z,Z*)-7,11-hexadecadienal (diene) lure] in plots treated with (*Z,Z,E*)-7,11,13-hexadecatrienal

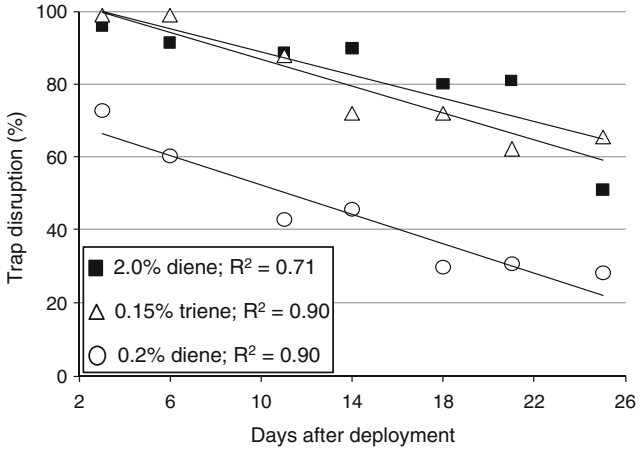

Fig. 4 Decay of trap disruption (%) over time of three SPLAT formulations containing single pheromone components: (*Z,Z*)-7,11-hexadecadienal (diene) or (*Z,Z,E*)-7,11,13-hexadecatrienal (triene). Disruption of trap catch was measured as the number of male *Phyllocnistis citrella* captured in traps baited with a 3:1 triene:diene lure in plots treated with a disruptant (diene or triene) expressed as a percent of the trap catch in the control plots

than traditional factorial designs (Lapointe et al. 2008) and, in the case of pheromone studies, can contribute insight into underlying mechanisms associated with mating disruption. Geometric multivariate approaches to mixture problems have wide applicability for experimental biology, as recently shown for insect diets (Lapointe et al. 2008), studies of ion-specific effects (Evens and Niedz 2008), and plant growth media (Niedz and Evens 2008).

Studies of disruption of moth species using various blends and concentrations of pheromone components, in general, have incompletely sampled experimental space defined by proportionality and amount (Charlton and Cardé 1981; Bellas and Bartell 1983; Flint and Merkle 1983; Moreira et al. 2006). We suggest that a more complete picture of the role of individual pheromone compounds in blends and their interactions, and important insight into mechanisms of mating disruption, can be obtained through use of non-confounded designs. Moreover, this approach minimizes the number of runs required in expensive and labor-intensive field trials that often are limiting for product development.

The natural ratio of two sex pheromone components (3:1 triene:diene) of *P. citrella* (Leal et al. 2006; Moreira et al. 2006) is a potent attractant in the field (Lapointe et al. 2006). In our study, attraction of males to baited traps declined markedly as the blend ratio departed from this natural ratio, with little interaction between proportion and amount (Fig. 2). Our geometric design surveyed the experimental space and produced a response surface that confirmed the work of Moreira et al. (2006) that the 3:1 blend is optimal for attraction at least over the release rates employed in this study.

The response surface generated in the disruption trial, however, was markedly different from that of the attraction trial. The straight line described by the response surface, corresponding to the highest pheromone concentration (0.2%) and X1-X2 axis (Fig. 3), demonstrated that trap shutdown increased in direct proportion to concentration of the triene with no synergistic or antagonistic blending effects between diene and triene. This lack of blending in the disruption trial suggests that an unnatural ratio consisting of one or the other of the single components could be as effective as the natural blend for mating disruption. This, together with the general lack of congruence between response surfaces, supports the conclusion of Stelinski et al. (2008) that disruption in this species is a non-competitive phenomenon.

The linear response of trap catch of *P. citrella* to individual components in binary blends deployed as disruptants may be analogous to the disorientation thresholds that define the "attraction area" according to the threshold hypothesis for pheromone perception proposed by Roelofs (1978). By applying Roelofs' "attraction area" concept to *P. citrella*, our results suggest that changes in binary proportions, away from the natural blend ratio, would produce a disorientation line with a steep slope for the diene and a much smaller slope for the triene. Considered this way, the amount of diene required to achieve disruption would be much greater than the amount of triene required, as demonstrated. Roelofs' threshold diagrams predict that, for species that utilize a binary pheromone mixture for upwind orientation, the natural blend should be most effective at low concentrations. Therefore, we might expect mating disruption to be more effective at lower release rates of the binary blend compared with either component alone. Our results with *P. citrella* do not support this extrapolation, at least within the concentrations tested in our investigation. Disruption, as measured in our field tests by reduced trap catch (Fig. 3), did not decline at low pheromone concentrations as the proportionality of the disruptant moved from the natural ratio to pure triene.

Under the competitive theory of mating disruption, males are predicted to follow false plumes associated with pheromone dispensers (Miller et al. 2006a). If disruption is non-competitive, no such orientation occurs. Typically, direct observation of male orientation to pheromone dispensers in the field is required to determine attraction. However, the response surfaces for *P. citrella* for attraction and disruption (Figs. 2, 3) demonstrate visually that the single triene component was equal to or greater in disruptive ability compared to the natural blend, even though males were not attracted to either component (diene or triene) singly. Effective trap shutdown was obtained with the natural blend (0.02%), the triene alone (0.015%), and

the diene alone (0.2%). Males of *P. citrella* were not attracted at close range in the field to SPLAT treatments that contained the natural blend or single component applications of SPLAT (LS, personal observation).

Therefore, our data suggest that disruption of male *P. citrella* occurred by a non-competitive mechanism (Stelinski et al. 2008). The non-competitive mechanisms of mating disruption are camouflage, desensitization, and sensory imbalance, as defined by Bartell (1982). Our data indicate that the 3:1 blend was highly attractive to males at a release rate approximating a female, whereas the triene or diene alone were not attractive. Based on current, as well as previous (Stelinski et al. 2008) research, we know that disruption of orientation by male *P. citrella* to traps by the 3:1 blend, triene alone, or diene alone occurs at certain concentrations of pheromone above the threshold for male activation and orientation. Furthermore, our data revealed that the triene is a superior disruptor of male *P. citrella* orientation compared with the 3:1 blend. Camouflage can be eliminated as a mechanism of disruption in our trials because, if it were operating, its effectiveness (i.e., disruption) should increase as the synthetic pheromone blend approximates the natural blend (Cardé 1990). Our results did not show such an effect (Fig. 3).

Desensitization, as a mechanism of disruption, can occur following prolonged exposure to an unnaturally high stimulation with synthetic pheromone, at the level of the peripheral (adaptation) or central (habituation) nervous system or both (Bartell 1982; Miller et al. 2006a). The two-component blend should be expected to cause greater desensitization of male *P. citrella* than the triene alone, because the blend acts on more receptors in total than does a single component. However, desensitization of a portion of receptors may be highly effective if it interferes with integration in the central nervous system. Thus, although our results indicate that disruption with the triene was superior to the 3:1 blend, desensitization as the mechanism of disruption cannot be ruled out. We believe that our field trapping and disruption results are most consistent with the sensory imbalance mechanism of disruption for *P. citrella*. Sensory imbalance involves males receiving a signal out of balance with the one their sensory system is optimized to perceive (Miller et al. 2006a). We found that the pure triene formulation was superior to the 3:1 blend in the disruption trial, and that we could disrupt trap catch with either pheromone component individually.

Minks and Cardé (1988) explored the question of whether optimal mating disruption of moths could be obtained through deployment of natural or unnatural ratios of pheromone components. They argued that available evidence suggested that natural blends should be most effective at the lowest dose and that, given the constraints of available information, pursuit of unnatural blends was unwise unless field trials demonstrated otherwise. Cardé and Minks (1995) reported that the majority of documented cases suggested that the natural blend provided best disruption at the lowest dose. As those authors note, none of the cases had been fully substantiated. We argue that with adequate experimental design and execution, optimal blend ratios and dosages for disruption can be identified and, further, may show that the natural blend may not be the most efficacious at low release rates. Furthermore, if the cost differential of synthesis of individual blend components is large, then economic considerations will drive practical decisions regarding product development for mating disruption applications. The case presented here for *P. citrella* demonstrates both of these points. First, the mixture-amount design allowed for exploration of the design space defined by proportionality and concentration of the two pheromone components. Second, the much lower cost of synthesis of the diene, compared to the triene, suggests that use of the diene, at a release rate an order of magnitude greater than that which would generate equivalent trap shutdown by the triene alone, may be warranted on economic grounds.

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compounds were washed off prior to the assay (Ruther et al. 2002; Roux et al. 2007). However, lifeless dummies are inadequate during bioassays when the range of responses from each interacting individual depends on the behavior of the others (Gamboa et al. 1991; Roulston et al. 2003).

A good way to avoid this problem consists of inserting live insects into a glass vial whose inner walls were previously coated with an extract. Although this method has been used successfully with honey bees, which rub their wings, thorax, and abdomen on the coated insides of the walls (Dani et al. 2005), the technique is not suitable for transferring semiochemicals to non-flying insects that walk on the walls.

There are non-destructive, nontoxic extraction techniques, such as solid phase micro extraction (SPME), which consists of gently rubbing the insects with a glass fiber coated with a membrane with an affinity for cuticular compounds. The fiber then is introduced into the gas chromatograph injector where the compounds are desorbed. Results obtained through this method are similar to those obtained with solvent extracts (Monnin et al. 1998; Turillazzi et al. 1998; Tentschert et al. 2002). Turillazzi et al. (1998) adapted this method by replacing the fiber with a clean piece of cotton wool or filter paper. After rubbing the insects with substrate, the compounds are rinsed from the cotton wool or filter paper with a solvent, and then analyzed. These extractions by "abrasion" allow the insects to be kept alive so as to monitor variations in their cuticular profiles at different stages of their lives; it provides results similar to those of solvent extracts (with some variations in relative proportions: Turillazzi et al. 1998). In addition to the cost and fragility of SPME fibers, it is difficult to use these techniques on small and/or fragile insects; moreover, the analyses must follow the extraction as quickly as possible because the time during which compounds can be conserved is limited. Adaptation with cotton wool allows some of these disadvantages to be avoided, but cotton wool is slightly less sensitive and can catch on spurs on the cuticle.

Another non-destructive, nontoxic extraction method involves rinsing individuals with water and using the aqueous extract in behavioral assays. Andrews (1911) noted that water-washed termites were attacked by nestmates as well as by alien individuals. Fresneau (1980) successfully transferred recognition signals in *Pachycondyla villosa*, a ponerine ant. By rinsing workers belonging to two different colonies, and then soaking them in baths already used to wash alien individuals, he was able to induce aggressiveness among nestmates and tolerance between alien workers. This method was used by Dejean et al. (1990) to test learning behavior in *P. villosa* workers that adopt a posture of prudence when encountering termite soldiers, but not termite workers. Rinsed termite workers soaked in the "bath" water of soldiers triggered a posture of prudence in ants; whereas, rinsed termite soldiers soaked in the "bath" water of workers did not induce any prudence, so that termite soldiers viciously bit the ants. The authors concluded that compounds, here serving as kairomones, were transferred (after emulsion) between the termite soldiers and workers.

Henderson et al. (1990) wanted to test several methods for removing nestmate discrimination pheromones and then suppressing aggressive behavior between alien *Formica montana*. The methods used included washing the ants with distilled water; however, this technique yielded poor results in comparison to hexane or pentane washes because water does not wash as well as "classical solvents." Indeed, after a water-based wash, many cuticular compounds remain on the insect cuticle, so this method does not always allow the aggressive behavior of non-nestmate individuals to be completely inhibited (O.R. pers. obs.). In these reports, the insects were rinsed by vigorously shaking them in vials half-filled with water to emulsify their cuticular hydrocarbons. The insects, at first numbed, recovered after a few minutes and could subsequently be used in bioassays. However, no chemical analyses then were performed to verify which compounds were transferred.

In this study, we tested the hypothesis that cuticular compounds can be extracted and transferred between ants by using water followed by analysis of the chemical composition of the extracts and behavioral assay of the ants' responses in the laboratory.

Methods and Materials

Insects The study was conducted with *Camponotus blandus* (Smith 1858) (Formicinae) and *Solenopsis saevissima* (Smith 1855) (Myrmicinae) (both Hymenoptera: Formicidae), two Neotropical species found frequently in open habitats. In October 2007 and February 2009, we gathered workers belonging to one colony from each species located about 20 m from each other along a forest trail situated near the Petit Saut dam (French Guiana).

Cuticular Compound Extraction Cuticular compounds were extracted by using two methods. First, twelve individuals from each species were freeze-killed, and the compounds extracted by placing the ants into 500 µl of hexane in a 2 ml glass vial for 5 min. The solvent was evaporated under a nitrogen stream, and the sample was re-suspended in 20 µl of hexane. Second, we used a method designed to allow the insects to be kept alive wherein thirty individuals were placed into a 30 ml glass vial along with about 20 ml of ultra pure water (34°C) and hand shaken vigorously for 5 min with the

aim of emulsifying the cuticular hydrocarbons. The ants were removed, and the aqueous emulsion was extracted with 500 µl of hexane by shaking the vials for 10 sec before allowing the contents to settle for 1 min. The hexane supernatant was transferred to a 2 ml glass vial, removed under a nitrogen stream, and the residue re-suspended in 20 µl of hexane for gas chromatographic-mass spectrometric (GC-MS) analysis.

Applying Cuticular Hydrocarbons to the Ants Camponotus blandus Workers were applied with the cuticular compounds of *S. saevissima* workers in two different ways. In both cases, about 70 workers from the two species were rinsed separately in 20 ml of water as described previously. The ants then were removed and placed into plastic boxes whose walls were coated with fluon® to prevent them from climbing out, and whose floor was lined with absorbent paper on which the ants dried. For the first application method, rinsed *C. blandus* were placed directly into the emulsion of *S. saevissima* cuticular compounds, shaken for 10 sec, and then left in the emulsion for 5 min. They then were removed and placed again into a plastic box where they were redried. To see how effectively the cuticular compounds were transferred, some individuals were freeze-killed, and their cuticular compounds extracted with hexane prior to GC-MS analysis. For the second application method, the water-based emulsion was extracted with hexane as previously described, and the residual hexane was carefully removed and the "bath" water transferred to a clean vial. Only then were the rinsed *C. blandus* placed into the water from which the hexane fraction (containing compounds with a greater affinity for hexane than water) was removed. This second technique was used to test the action of the aqueous emulsion alone.

Chemical Analysis Chemical analysis and the identification of compounds were conducted with a Finnigan Trace GC-MS 2000 Series chromatograph directly coupled to a mass spectrometer quadrupole detector. The entire system was operated by using the Xcalibur data system, version 1.2. MS spectra were recorded in the EI mode (70 eV) over a mass range of 50–550 mass units with two scans per second. One µl was injected into the splitless mode with an injector temperature of 280°C and a detector temperature of 300°C. An apolar Rtx®-5MS-MS capillary column (30 m×0.25 mm i.d.× 0.25 µm film thickness, 5% diphenyl and 95% dimethylpolysiloxane) was used. Elution was carried out with helium at 1 ml/min. The oven temperature was programmed from 80°C (for 30 sec) to 200°C at 20°C/min, from 200°C to 240°C at 3°C/min, from 240°C to 300°C at 30°C/min, and left at 300°C for 19 min. As it is important only to check if the compounds in the water-based extract are the same as in the hexane-based extract, only tentative identifications were made by comparing them with a spectral database, retention indices, a series of standard linear alkanes, and the calculation of methyl positions on the basis of mass spectra. Then, these tentative identifications of the compounds were provided for informational purposes only; some were cross-checked with mass spectra found in the literature. Then, the alkaloids were identified based on comparisons with the work of MacConnell et al. (1971), Brand et al. (1972) and, more recently, Chen and Fadamiro (2009a, b). Some methylalkanes were identified through comparisons with the results obtained by Nelson et al. (1980) on the cuticular methylalkanes of some *Solenopsis* species.

Behavioral Assays To test the effectiveness of our extraction and application methods, five sets of ants were prepared: (1) Untreated *C. blandus* workers; (2) untreated *S. saevissima* workers; (3) *C. blandus* workers with the application method 1 thought to contain the cuticular extracts of *S. saevissima* workers; (4) *C. blandus* workers with application method 2 consisting of the water-based extract of the *S. saevissima* workers without compounds soluble in hexane; and (5) "blank" *C. blandus* workers, i.e., individuals that were rinsed in two different baths of clean water.

Behavioral interactions were tested between the following workers: (1) untreated *C. blandus vs*. untreated *C. blandus*; (2) untreated *C. blandus vs*. "blank" *C. blandus*; (3) untreated *C. blandus vs. C. blandus* with application method 2; (4) untreated *C. blandus vs. C. blandus* with application method 1; (5) untreated *S. saevissima vs*. *C. blandus* with application method 1; and (6) untreated *C. blandus vs*. untreated *S. saevissima*. All tested *C. blandus* workers belonged to the same colony. For each behavioral interaction, two workers were placed together for 5 min in a neutral arena (a Petri dish; 60 mm diam and 20 mm deep) whose walls were coated with Fluon® to prevent the ants from climbing out. We scored the interactions as follows: 1 = antennal contact (physical contact, but no aggressive response; may include trophallaxis), 2 = escape (sudden U-turn and running quickly), 3 = aggressiveness (brief biting by one or both of the workers; may include jerking and opening the mandibles), and 4 = fighting (prolonged aggressiveness, including prolonged biting and stinging [*Solenopsis*] or venom spraying [*Camponotus*]). We repeated each kind of confrontation 60 times, keeping the highest value noted for each series of 5-min observations, and used each worker only once. Because *S. saevissima* is active around the clock and *C. blandus* is typically diurnal (Orivel and Dejean 2002), all bioassays were performed during the day.

Statistical Analyses Levels of aggressiveness between interacting ants were compared by using the Kruskal–Wallis test. A *post-hoc* test (Dunn's multiple pairwise comparisons test) was then performed to isolate the dyadic interactions that differed from the others. All statistical analyses were performed with GraphPad Prism 4.03 software (GraphPad Software Inc 2005).

Results

Chemical Analysis and Extraction Methods The two methods of extraction produced both quantitatively (in terms of proportions) and qualitatively similar GC-MS profiles for both ant species (Fig. 1).

The GC-MS profiles of the two ant species were different (Fig. 1a–d). The GC-MS profiles for *C. blandus* contained 23 compounds, all hydrocarbons, ranging from C27 to C40 (Table 1). The *S. saevissima* profile had 34 compounds; the eight early eluting compounds, representing the bulk of the extracts, were piperidine alkaloids typical of *Solenopsis* venom (MacConnell et al. 1971; Brand et al. 1972). The later eluting compounds were all hydrocarbons ranging from C23 to C32. Only heptacosane (peak 26) was common to both species, but present at trace levels.

Application Methods The gas chromatogram obtained from rinsed *C. blandus* treated with the emulsion of *S. saevissima* cuticular compounds was not a perfect blend of the cuticular signatures of the two species (Fig. 1e). The *S. saevissima* alkaloids were transferred poorly to the rinsed

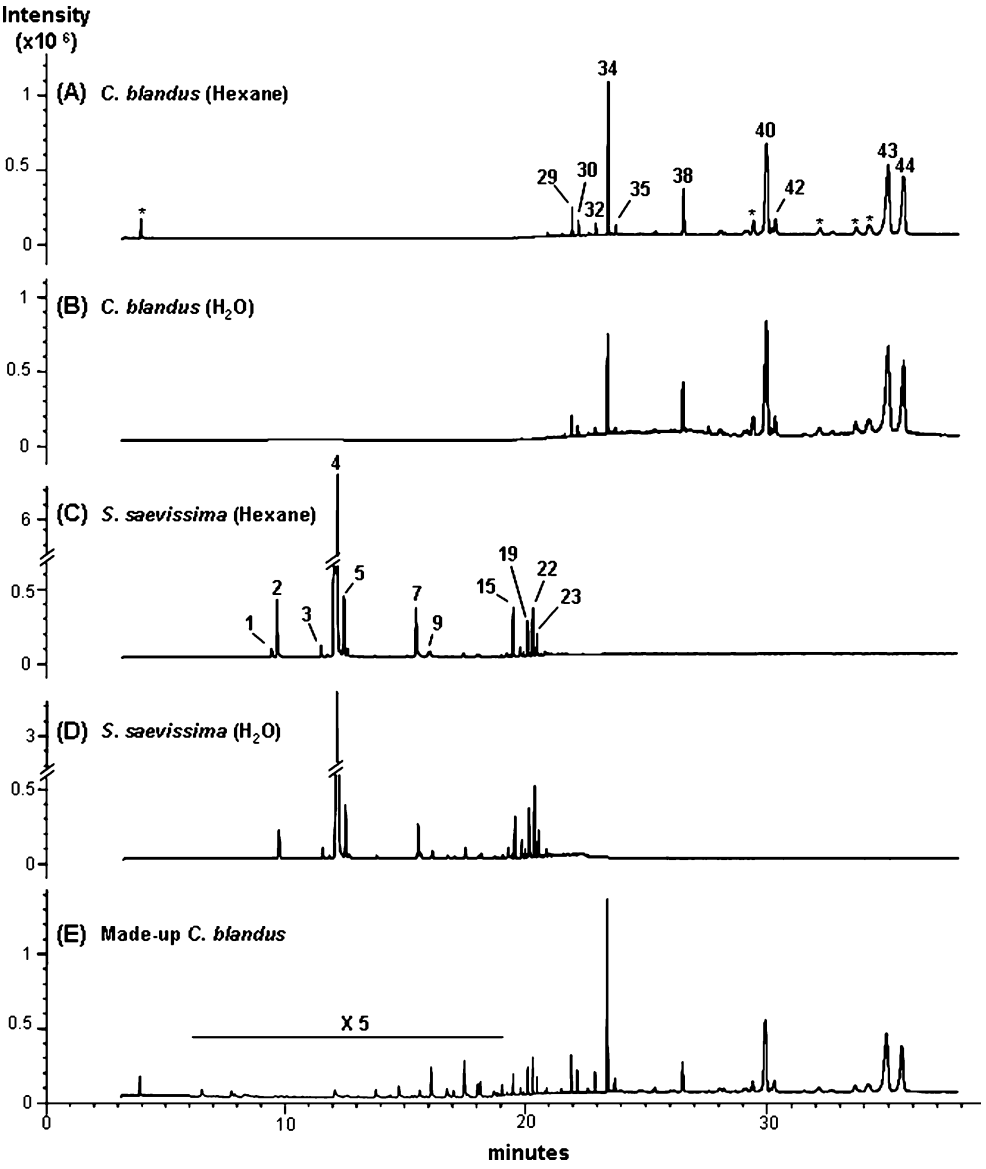

Fig. 1 Gas chromatographic-mass spectrometric traces of extracts from *Camponotus blandus* and *Solenopsis saevissima* workers. Analyses were conducted on extracts prepared by two methods (**a**–**d**), as well as on a hexane-based extract from *C. blandus* workers "made-up" with the emulsion of *S. saevissima* cuticular compounds (**e**); the area under the horizontal line is enlarged five times; * = contamination; see Table 1 for peak assignments

C. blandus, whereas all of the hydrocarbons from *S. saevissima* (peaks 9–27) were transferred efficiently, and the proportions of the transferred hydrocarbon components were comparable to those in the native extracts in most cases (except for compounds 15, 19, 22, and 23, which were recovered in smaller proportions than in the extracts of the worker *S. saevissima*, Table 1).

Behavioral Assays We typically noted the absence of aggressiveness (level 1; i.e., antennation and trophallaxis; Fig. 2) among untreated *C. blandus* nestmates that is significantly different from the high level of aggressiveness that occurred among untreated *C. blandus* and untreated *S. saevissima* workers (mean aggressiveness levels: 1±0 and 2.46±0.09, respectively; $P<0.001$; Fig. 2). The latter triggered some fighting and they mainly raised and vibrated their gaster ("flagging"), a behavior corresponding to the airborne dispersal of venom (see Obin and Vander Meer 1985). As a result, *C. blandus* workers ran, jerked away, or fought when contact became frequent. Interactions between untreated and "blank" *C. blandus* (to test the effect of double rinsing) were not significantly different from those observed between untreated nestmates (mean aggressiveness levels: 1±0 and 1.06±0.04, respectively; *NS*; Fig. 2). Inversely, interactions between untreated *C. blandus* workers and those with the "S make-up" resulted in levels of aggressiveness significantly higher than the two previous situations involving *C. blandus* workers (mean aggressiveness levels: 1±0 and 2.15±0.13, respectively; $P<0.001$; Fig. 2). Nevertheless, interactions between untreated *C. blandus* workers and *C. blandus* workers with "S-H make-up" were not different from interactions with untreated or "blank" *C. blandus* workers (Fig. 2). The interactions between untreated *C. blandus* workers and *C. blandus* workers with application method 1 were not significantly different from interactions between untreated *C. blandus* workers and untreated *S. saevissima* workers (mean aggressiveness levels: 2.15±0.13 and 2.46±0.09, respectively; *NS*; Fig. 2). Also, interactions between untreated *S. saevissima* workers and *C. blandus* workers with application method 1 were not significantly different from those between *C. blandus* workers and *C. blandus* workers with application method 1 (mean aggressiveness levels: 2±0.15 and 2.15±0.13, respectively; *NS*; Fig. 2), but they were different from those between untreated *C. blandus* and untreated *S. saevissima* workers (mean aggressiveness levels: 2±0.15 and 2.46±0.09, respectively; $P<0.001$; Fig. 2). Untreated *C. blandus* workers mainly ran away after encountering *S. saevissima* workers as well as *C. blandus* workers with the application method 1 (level 2 of aggressiveness), but *C. blandus* workers with the application method 1 did not run away when they encountered untreated *S. saevissima* workers (behavioral observations not shown).

Discussion

Our study demonstrates that it is possible to extract cuticular compounds from live tropical ants by using a tepid water bath, and that the extracted workers remain active even after being rinsed twice. Also, this method permits the cuticular extracts to be reapplied onto other live tropical ants with the aim, as in our study, of transferring a part or all of the cuticular recognition signals.

That ants living in equatorial countries survived our rinsing treatment is not surprising as they are adapted to living in areas with heavy rain, particularly ground-nesting species whose nests can be flooded. When flooded, the colonies of several species form rafts that float on the flooding waters. This frequently has been reported for fire ants (*Solenopsis invicta*, *S. saevissima*, and *Wasmannia auropunctata*), *Pheidole* spp. (all Myrmicinae) and the Argentine ant (*Linepithema humile*; Dolichoderinae) (Hölldobler and Wilson 1990; Morrison 1998; Holway et al. 2002; Haight 2006).

Water is a polar solvent and so is not theoretically adapted to extracting non-polar compounds. It also is well known that cuticular lipids are mainly made up of hydrocarbons, which are not water-soluble compounds. Nevertheless, our study has shown that by "relatively vigorously" shaking the glass vial containing the water and the ants, tepid water can be used to extract cuticular hydrocarbons in a way that is similar to using hexane. In fact, these non-polar compounds were not solubilized, but emulsified and floating on the surface of the water. To explain how the emulsion can transfer cuticular lipids on the ants, Dejean et al. (1990) used the comparison of birds caught in an oil slick, with the cuticular lipids acting as the oily film on the surface of the water and the live ants as the birds that were coated with the "pollutant." Even if water cannot extract all of the cuticular lipids (in terms of quantity), it is powerful enough to extract part of them, and to produce qualitatively the same extracts as hexane; moreover, the quantity extracted and reapplied is enough to induce the expected behaviors. In addition, the extraction of cuticular compounds from the "bath" water of *S. saevissima* workers by adding hexane prior to applying the cuticular compounds of *C. blandus* (application method 2) showed that the transferred signal really is contained in the hexane fraction and could be hydrocarbons.

We point out that the volume of water used in the application experiment is less important than the diameter of the vial. Indeed, as we were dealing with an emulsion,

Table 1 Tentative identification and relative quantities[a] of cuticular compounds from *Camponotus blandus* and *Solenopsis saevissima* by using two different methods of extraction and for application of *S. saevissima* cuticular extract to *C. blandus* workers

Peak	Retention index	*C. blandus*		*S. saevissima*		Applied extract	Identification	m/z
		Hexane	H_2O	Hexane	H_2O			
1	1851			+	+	tr	2-Methyl-6-(4-undecenyl)piperidine	98-111-124-180-236-251
2	1871			+ +	+ +	tr	2-Methyl-6-(undecyl)piperidine	98-238-252
3	2016			+	+	tr	2-Methyl-6-(4-tridecenyl)piperidine	98-111-124-180-264-278
4	2069			+ + +	+ + +	tr	2-Methyl-6-(4-tridecenyl)piperidine (isomer of peak 3)	98-111-124-180-264-278
5	2081			+ +	+ +	tr	2-Methyl-6-(tridecyl)piperidine	98-266-280
6	2094			+	+	tr	2-Methyl-6-(4,x-tridecadienyl)piperidine	98-111-124-178-220-262-276
7	2271			+ +	+ +	tr	2-Methyl-6-(4-pentadecenyl)piperidine	98-154-208-292-307
8	2292			+	+	tr	2-Methyl-6-(pentadecyl)piperidine	98-294-308
9	2300			+	+	+	*n*-Tricosane (*n*-C_{23})	-
10	2332			tr	tr	tr	11-Methyltricosane (11-meC_{23})	168/169-196/197
11	2370			+	+	+	3- Methyltricosane + unknown (3-meC_{23} + unk.)	56/57-308/309
12	2400			+	+	+	*n*-Tetracosane (*n*-C_{24})	-
13	2407			+	+	+	3,7-Dimethyltricosane (3,7-dimeC_{23})	57-127-253-323
14	2471			+	+	+	Pentacosene ($C_{25:1}$)	-
15	2500			+ +	+ +	+	*n*-Pentacosane (*n*-C_{25})	-
16	2536			+	+	+	13- and 11-Methylpentacosane (13 + 11-meC_{25})	168/167-196/197-224/225
17	2538			tr	tr	tr	9-Methylpentacosane (9-meC_{25})	140/141-252/253
18	2557			tr	tr	tr	5-Methylpentacosane (5-meC_{25})	84/85-308/309
19	2578			+ +	+ +	+	3-Methylpentacosane (3-meC_{25})	56/57-336/337
20	2590			tr	tr	tr	5,9-Dimethylpentacosane (5,9-dimeC_{25})	85-155-253-323
21	2600			tr	tr	tr	*n*-Hexacosane (*n*-C_{26})	-
22	2620			+ +	+ +	+	3,7- 3,9- and 3,11-Dimethylpentacosane (3,7 +3,9+3,11-dimeC_{25})	56-127-155-183-225-253-281-351
23	2633			+ +	+ +	+	3,7,11-Trimethylpentacosane (3,7,11-dimeC_{25})	127-197-224-295
24	2666	tr	tr			tr	Unknown branched hydrocarbon	141-281
25	2694			+	+	+	4,14- and 4,18-Dimethylhexacosane (4,14+4,18-dimeC_{26})	70-141-197-225-281-351
26	2700	tr	tr	tr	tr	tr	*n*-Heptacosane (*n*-C_{27})	-
27	2728			tr	tr	tr	Unknown	140/141-210/211-280/281
28	2800	tr	tr			tr	*n*-Octacosane (*n*-C_{28})	-
29	2844	+	+			+	4-Methyloctacosane (4-meC_{28})	70/71-364/365
30	2900	+	+			+	*n*-Nonacosane (*n*-C_{29})	-
31	2976	tr	tr			tr	4-Methylnonacosane + Unknown (4-meC_{29} + unk.)	70/71-378/379
32	3000	+	+			+	*n*-Triacontane (*n*-C_{30})	-
33	3040			tr	tr	tr	Unknown branched hydrocarbon	197-224-253-281
34	3073	+ + +	+ +			+ + +	4-Methyltriacontane + unknown (4-meC_{30})	70-155-309-393
35	3100	+	+			+	*n*-Hentriacontane (*n*-C_{31})	-
36	3200	tr	tr			tr	*n*-Dotriacontane(*n*-C_{32})	-
37	3260	tr	tr			tr	Unknown	197-253-281-421
38	3356	+ +	+ +			+ +	17-Methyltritriacontane (17-meC_{33})	252/253
39	3457	+	+			+	16-Methyltetratriacontane (16-meC_{34})	238/239-280/281

Table 1 (continued)

Peak	Retention index	*C. blandus*		*S. saevissima*		Applied extract	Identification	m/z
		Hexane	H_2O	Hexane	H_2O			
40	3566	+ +	+ + +			+ +	19-Methylpentatriacontane (19-meC_{35})	280/281-252/253
							and 13,21-Dimethylpentatriacontane (13,21-dimeC_{35})	196/225/323/351
41	3575	tr	tr			tr	Unknown	-
42	3600	+	+			+	*n*-Hexatriacontane (*n*-C_{36})	-
43	3786	+ +	+ +			+ +	mixed of unknown branched hydrocarbons	197-253-323-379
44	3808	+ +	+ +			+ +	17-Methyloctatriacontane (17-meC_{38}) + unknown branched hydrocarbons	196-253-267-323-393

tr traces; *x* unknown position of the double bond; *m/z* mass-to-charge ratio

[a] +++ = base peak (100%); ++ = peak height>10%; + = peak height<10%

the smaller the diameter of the vial, the greater the height of the layer of the supernatant, facilitating the transfer of compounds to the ant cuticle. In the choice of the vial, then, there is a trade-off between diameter and the volume of water able to contain, in this case, 70 ants.

The main problem with using live insects in a water or polar solvent rinse is that, in addition to the expected cuticular hydrocarbons, extracts can contain exocrine gland secretions and regurgitated and excreted items. However, Vander Meer and Morel (1998) pointed out that solvent extracts of dead insects also contain such compounds. In our study, cuticular hydrocarbons and alkaloids from the venom gland of *S. saevissima* were obtained through hexane- and water-based extractions. Like hydrocarbons, fire ant alkaloids are not water-soluble and less dense than water (Blum et al. 1958). Nevertheless, they were poorly transferred to the *C. blandus* cuticle, probably due to their greater polarity.

We noted that during the interactions involving *S. saevissima* or *C. blandus* workers with the application method 1, untreated *C. blandus* workers ran away more frequently in the former than in the latter case (70% *vs*. 36.7%). This can be due to the alkaloids contained in the *S. saevissima* venom that have a repellent function during heterospecific encounters (see Obin and Vander Meer 1985, for *S. invicta*). Indeed, that the alkaloids are not transferred during the reapplication process could explain at least a part of the difference. Also, because aggressive interactions are potential sources of injury or death, ants rather initiate fights when they must defend their nest, territories or food (Roulston et al. 2003). So, in a neutral arena devoid of food

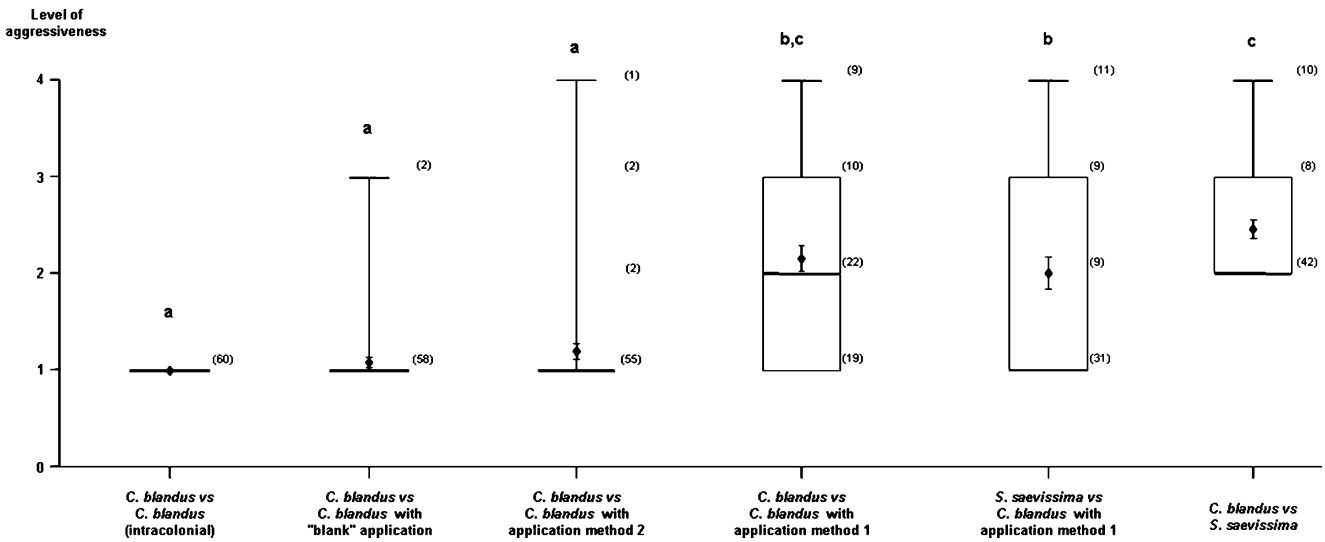

Fig. 2 Box plot and mean ± se of aggressiveness responses observed during the six different types of interactions between *Camponotus blandus* and *Solenopsis saevissima*. Mean (♦). The *box* plots indicate the median (*large horizontal bars*), the 25th and 75th percentiles (*white squares*), and the minimum and maximum values (whiskers). Statistical comparisons were performed by using the Kruskal–Wallis test (H=186.7; df=5; P<0.001) followed by a Dunn's *post hoc* test: different *letters* indicate significant differences (P<0.001). (n) = number of occurrence for each level of aggressiveness

sources, it is in the protagonists' best interest to avoid conflict. The non-aggressive behavior observed during interactions between untreated *C. blandus* workers and *C. blandus* workers with the application method 1 could result from the imperfect distribution of the cuticular compounds on the cuticle of the reapplied workers (Ruther et al. 1998). However, during interactions between untreated *S. saevissima* workers and *C. blandus* workers with the application method 1, reapplied *C. blandus* workers ran away less often than in the previous cases. This could be because its perception of its own "gestalt odor" was altered and partially matched the odor of *S. saevissima* workers. Fresneau (1980) found that this may lead to tolerance of the alien during intraspecific confrontations.

In summary, we have shown that using water-based extracts of cuticular compounds is similar to using hexane-based extracts, and that they can be reapplied onto other individuals through soaking, all while keeping the individuals alive. This method of transferring cuticular compounds might be applicable to other insect-insect or even insect-plant interactions.

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also is assumed that the level of area marking could inform the ants about their distance from the nest since in central place foragers, the density of exploring nestmates and thereby the density of home-range marks decrease with distance (Devigne and Detrain 2006). Based on the information provided by the level of home-range marking, ants fine tune their recruitment and foraging behavior. By increasing the trail intensity as well as the rate of information transfer on heavily marked areas, scouts reduce the number of foragers mobilized to less frequented areas that are potentially dangerous and promote recruitment and exploitation of food sources to better known sites (Devigne et al. 2004). The nest odor is important when the colony has to emigrate: ants will always prefer to settle in a marked nest than in an unmarked one (e.g., *Lasius niger* (Depickère et al. 2004); *Temnothorax albipennis* (Franks et al. 2007)).

In this paper, we investigated whether area marking in *Lasius niger* ant species originates from cuticular hydrocarbons and to what extent they show the same level of colony specificity as cuticular recognition cues over the ant body. First, we analyzed the cuticular hydrocarbons (CHCs) of forager workers from different localities in France to investigate geographical variations in the cuticular chemical profile of this species. Nestmate recognition in *Lasius* is highly efficient with workers also being able to discriminate nestmates from non-nestmates (Akino and Yamaoka 2005). Therefore, we predicted that the monogynous *L. niger* ant has a well differentiated colonial cuticular profile. Second, we compared the cuticular profiles of head plus thorax vs. legs alone in order to determine whether legs have a secretory role, as hypothesized for Japanese *Lasius* species (Yamaoka and Akino 1994). In this case, thorax and legs would show quite different chemical profiles. In contrast, they would be similar if there is a continuous update of the profile of the individuals due to self-grooming. Third, we analyzed the chemical profile of traces left over inner walls of ant nests, as well as those deposited by the foraging workers over the areas surrounding the nest. We predicted that the inside-nest marking would be colony-specific. Since ants behave similarly over areas that are marked by an alien or by their own colony (Devigne and Detrain 2002), we predicted that, outside the nest, there would be a less clear-cut colonial identity of traces left over the substrate of foraged areas.

Methods and Materials

The Ants The black garden ant, *Lasius niger*, is common in temperate European regions. Colonies were collected in May and June 2007 at three locations: near the cities of Tours (Azay sur Cher, 0° 48′ E, 47° 19′ N, 80 m asl, orchard, colonies T5, T6, and T7) and Pau (Sauvagnon, 0° 22′ W, 43° 25′ N, 187 m, garden, colonies S2 and S3), and in the Alps (M: Morillon, Haute-Savoie, 06° 41′ E, 46° 05′ N, 670 m, on the edge of a torrent, one colony). Within localities, colonies were separated by less than 10 m. All fragments of colonies were queenless and composed of 300 to 500 workers with brood. We checked that the ants were distinct from the close species *L. alienus* and *L. platythorax* by examining their morphological characters as well as their cuticular hydrocarbon profiles which were different (A.L. unpublished).

Colonies were kept in the laboratory (temperature 25°C, natural daylight), in artificial nests composed of two plastic containers (20×20×10 cm) connected by a bridge. The first box contained the nest made with glass tubes half filled with water and covered with black paper. The second container provided a foraging area to the colony, in which twice a week we offered *Tenebrio molitor* larvae and commercial bumblebee solution (Bee Happy®).

Chemical Analyses To study cuticular hydrocarbons, samples consisted of extracts of head plus thorax with the legs. Gaster was eliminated to prevent contamination from Dufour gland. We pooled five workers to get a concentrated sample. To determine a possible secretory role of legs, samples were made of tibias and tarsae from 20 workers. All workers were foragers. Ants were immersed in 200 µl of pentane for 5 min. Samples were removed, and 5 µl of pentane containing 50 ng of eicosane (C20) were added as an internal standard. For analyses, the solvent was evaporated until 10 µl remained. Three µl were injected into a FID gas–chromatograph (VGM250Q system, Perkin-Elmer) with a split/splitless injector and flame ionization detector that used a DB-5 fused silica capillary column. Temperature was kept at 150°C during the initial splitless 2 min, raised from 150°C to 300°C at 5°C/min, and held at 300°C for the last 10 min. The non volatile cuticular lipids were identified with the same GC coupled to a Perkin-Elmer MS operating 70 EV. A series of linear hydrocarbon standards (C20, C22, C24 … to C40) were injected regularly in order to have references. We also injected one cuticular extract into a high temperature column (DB-5HT) to 370°C to check if some hydrocarbons with high molecular weights appeared, as has been found in some ants [see (d'Ettorre and Lenoir 2009)]. As no more hydrocarbons were detected, we subsequently used the normal DB-5 column. The areas of peaks were measured by peak integration with a Perkin Elmer Turbo-Chrome Workstation, and relative proportions were calculated. The quantities of compounds were calculated by using the internal standard areas (ng per head + thorax or per six legs, i.e., one ant).

Chemical compounds deposited on the substrate by the ants were analyzed by using the SPME technique. A polydimethylsiloxane (PDMS) 7 µ (fused silica/SS) fiber

was rubbed on substrate for 5 min. The fiber was desorbed in the GC under the same conditions as for liquid extracts. Three locations were chosen to analyze deposited chemicals marks: inner nest walls, area in front of the nest entrance, and foraging area. For the inside nest marking, we gently pushed the ants outside the nest and rubbed the fiber over the inner walls of the glass tubes. To collect area marking, glass slides were washed completely with a detergent and rinsed several times with hexane. We checked with SPME that the slides were free of any detectable chemical traces. These clean slides were deposited just in front of the nest entrance and in the foraging arena. Since preliminary trials showed that slides in the foraging arena were frequently covered with refuse particles, we put them for at least 2 days on the bridge between the two containers where they were kept clean. It has been shown that the profiles obtained with SPME and classical solvent extraction are qualitatively identical (Sledge et al. 2000; Tentschert et al. 2002), but a precise quantitative analysis shows that the proportions of compounds are slightly different. So, in order to get comparable data for the cuticle and the substrate marking, we also performed a 5 min SPME on the thorax of ants killed in the refrigerator. Moreover, we deposited in five samples a standard of 100 ng of C20 on the fiber to estimate the quantities retrieved by the SPME technique.

Statistics We compared the average relative amounts of different hydrocarbon classes by using a non-parametric Kruskal–Wallis test. To analyze the grouping of samples, we performed discriminant analyses on the peaks present in more than 1% amount followed by post-hoc F tests. We did not transform the data to compensate for their non-independence, since it introduces additional background noise and gives similar results. We also analyzed the distances between groups after discriminant analyses by using the Squared Mahalanobis distances (SMD), and we compared them by using the Kruskal–Wallis non-parametric test. To compare the hydrocarbon quantities we used Mann–Whitney *U* test.

Results

Cuticular Hydrocarbons and Geographical Variations *Lasius niger* workers show a wide spectrum of hydrocarbons from 25 to 39 carbons. These are composed of saturated n-alkanes (3.67%±1.39; mean ± SD, N=46) and corresponding alkenes (1.90%±0.72), large quantities of monomethyl-alkanes (29.70%±4.73), dimethyl-alkanes (58.05% ± 4.58), and trimethyl-alkanes (6.37%±2.64) (Fig. S1 and Table S1). Twenty two peaks are present in more than 1%, the two major peaks are a mixture of methyl-C31 (peak n° 30: 9+11+13+15Me-C31; 11.41%± 3.62) and a mixture of dimethyl-C31 (peak n° 32: 9,11+ 9,13+9,15DiMe-C31; 20.83%±3.77). The total hydrocarbon quantity is estimated at 521±396 ng/head + thorax + legs (N=21).

Discriminant analysis indicates good discrimination among the samples. Among the six colonies, four are well discriminated (T6, T7, S3 and S2, with all P<0.004, *Post-hoc* test F). Colonies M (Morillon) and T5 (Tours 5), however, are not differentiated (P=0.35) (Fig. 1). In the same locality (three colonies from Tours and two colonies from Pau), colonies are clearly differentiated. The Squared Mahalanobis Distances (SMD) are different between the groups (Kruskal–Wallis H(2, N=174)=85.5, P<0.01). The intracolony variation (among individuals) is small (19.89±2.79, mean ± SD, N=35). In contrast, intercolonial SMD between neighboring colonies in the same site is high (1917.14± 939.53, N=34; P<0.001 compared to intracolonial variation). This distance is not higher when we consider two colonies from different locations (for example Tours and Pau): 1666.42±1061.21, N=105 (P=0.46 NS compared to intercolonial variation in the same habitat).

These results indicate that in *L. niger*, there is a colonial chemical identity with colonies that inhabit the same habitat, being well differentiated in distances of a few meters. Additionally, the geographical variation is weak for this species since the SMD distance between the three sites is of the same order of magnitude as those found in the same habitat (one colony from the Alps has the same profile as one from Tours).

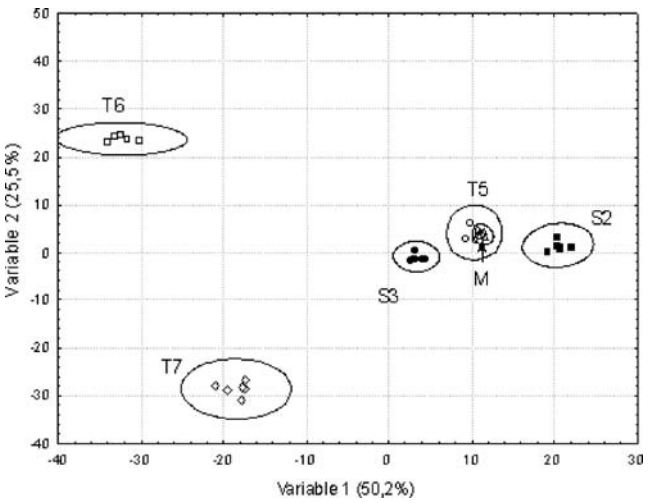

Fig. 1 Discriminant analysis of *Lasius niger* cuticular hydrocarbons. T = Tours (Azay sur Cher, colonies T5, T6, and T7); S = Sauvagnon near Pau (colonies S2 and S3); M = Morillon (Alps). Lambda Wilk< 0.001, F (120, 34)=21.68, P<0.001. *Ellipses* are 95% confidence intervals

Hydrocarbons on the Legs Legs contained the same hydrocarbons as the body cuticle (Table S1): all peaks were present and no new peak appeared. The hydrocarbon quantities were, however, much smaller on the legs (16.4 ng±10.8/worker, N=17, i.e., 3.15% of the thorax plus head cuticle content and less than 3 ng per each tibia + tarsus).

A discriminant analysis of hydrocarbons present on bodies or legs performed on the three colonies confirmed the high segregation among colonies seen in Fig. 1. It also shows that legs bear the colony profile (all P<0.001, F *post-hoc* test; see Fig. S2). By making a new analysis and using as discriminant variables the body part (head + thorax or legs) and the colony, we found that all groups are separated (all P<0.001, F *post-hoc* test) (Fig. 2). A main separation appears on the first axis (76.5% of the variance) among the three colonies. The discrimination is significant but less pronounced between the body cuticles and the legs within the same colony (12.9% of the variance). It indicates that the leg's cuticular profile is slightly different from the body one. It is characterized by an increase of the relative amount of n-alkanes (3.67%±1.39 for cuticle vs. 13.34± 6.69 for legs; H (1, n=63)=34.81, P<0.001), trimethyl-alkanes (6.37%±2.64 vs. 12.74±4.76; H=25.17, P<0.001) and alkenes (1.90%±0.72 vs. 5.73±5.10, H=4.37, P= 0.04). This increase is not due to mono-methyl-alkanes (29.70%±4.73 vs. 29.78±4.65; H=0.54, P=0.82) but to a decrease of dimethyl-alkanes (58.05%±4.58 vs. 38.41± 7.99; h=33.72, P<0.001) (see Table S1). There are no differences between the lengths of hydrocarbon chains: all n-alkanes from C25 to C31 increased and most dimethyl-alkanes decreased.

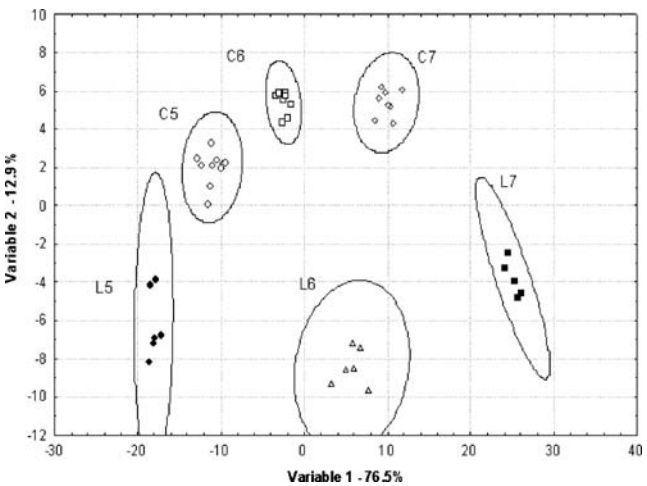

Fig. 2 Discriminant analysis of the hydrocarbons extracted from body cuticle (C) and legs (L) of colonies 5, 6, and 7. Lambda Wilk<0.001, F (130,64)=10.097, P<0.001. *Ellipses* are 95% confidence intervals

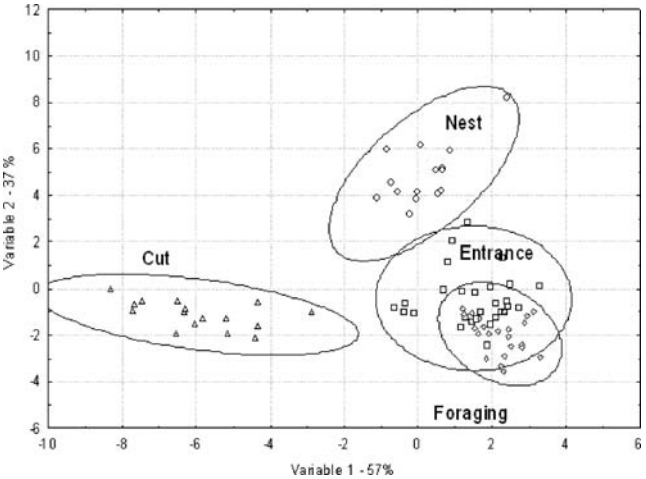

Fig. 3 Discriminant analysis of hydrocarbons obtained by SPME from cuticle (*Cut*), inner walls of the nest (*Nest*), entrance of the nest (*Entrance*) and foraging arena (*Foraging*), independently of the colonies which are not separated. Lambda Wilk 0.008, F (87, 150)= 7.450, P<0.001. *Ellipses* are 95% confidence interval

Hydrocarbons Obtained by SPME SPME and washing of the cuticle gave the same profiles, and the proportions of compounds are similar [Nei index of similarity between the two profiles = 0.96, i.e., comparable to intracolonial variation, see for example (Nowbahari et al. 1990; Lenoir et al. 2001a)]. This means that SPME and washing data can be compared directly.

In a first analysis, we compared the SPME chemical profiles of thorax cuticle, inner walls of the nest, nest entrance area and foraging arena independent of the colony (Fig. 3). The first axis separates the thorax cuticle from the deposited substances, indicating a clear distinction between them. The second axis separates the nest from the entrance and the arena. All groups are statistically different, but 8.5% of the values are not well classified in the foraging and entrance areas with Mahalanobis distances between them being much smaller (8.38 vs. all other distances >33). This indicates a clear separation between the cuticle and the substrate markings. Additionally, the marks left on the inner walls are different from the entrance and the foraging areas, the two latter being not very different. Globally, there is a gradient from the nest to the outside: the relative quantities of n-alkanes increase (from 6.58% to 29.36%), due mainly to a decrease in dimethyl-alkanes and alkenes. The proportions of methyl-alkanes and trimethyl-alkanes do not change (Fig. 4).

The amounts of hydrocarbons retrieved during 5 min of SPME rubbing are variable, but always higher when extracted from the nest walls than from the entrance, or foraging areas: 71.0 ng±80.07, N=15 for the nest, 15.56 ng ±21.45, N=28 for the entrance and 17.12 ng±14.98, N=23 for the foraging area (U Mann–Whitney N/E: P=0.005; N/ F: P=0.02 and E/F: P=0.28). These values are only

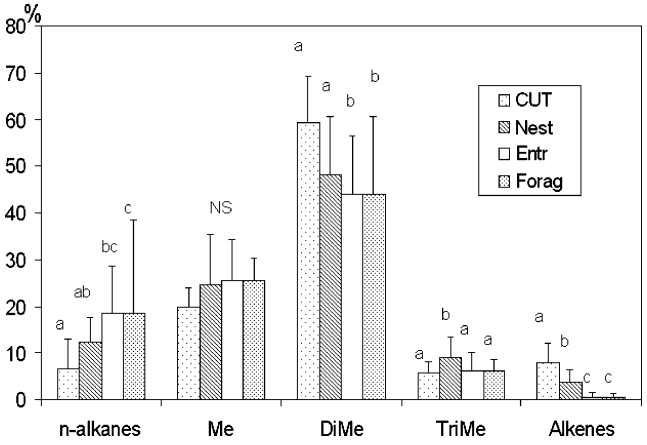

Fig. 4 Relative quantities of hydrocarbon classes present on the thorax cuticle or deposited at different locations (mean ± SD). Different letters indicate significant differences (Kruskal–Wallis test). Me = monomethyl-alkanes, DiMe = dimethyl-alkanes, TriMe = trimethyl-alkanes

comparative, and indicate that the hydrocarbons are more concentrated on the nest walls.

In a second analysis, we performed a discriminant analysis on all the SPME data for the deposited marks: inside-nest walls, entrance, and foraging areas (Fig. 5). The first axis (46.1%) showed discrimination between marking of the nest walls and those laid over areas outside the nest. The distances for inside nest marks were significant between N5/N6 (P=0.004) and N5/N7 (P=0.002) but not between N6 and N7 (P=0.30). This suggests that inside the nest, discrimination is not absolute, and errors are likely to occur. The situation also is complex concerning markings of the nest entrance and foraging arena. Colony 7 is well differentiated from colonies 5 and 6, but there is poor discrimination between its nest entrance and its foraging area (E7 / F7, P=0.04). Colonies 5 and 6 are not at all discriminated (all P>0.05, with a maximum for E6 / F6, P=0.85).

In a third step, considering that methyl branched hydrocarbons could be more important in nestmate recognition [see (Dani et al. 2001) for wasps, reviews by (Hefetz 2007; d'Ettorre and Lenoir 2009), and recent data on some species of *Formica* (Martin et al. 2008a)], we performed a discriminant analysis that used only mono-, di- and trimethyl-branched hydrocarbons. It does not change the results for nest marking: the two colonies 5 and 6 are not discriminated showing a low degree of differentiation for the inside nest marking (data not shown). By contrast, as regards entrance and foraging marks (Fig. 6), a good discrimination appears among the three colonies (only the distance in foraging arenas between colony 5 and 7 is at the significance limit with P=0.05). Nevertheless, the marks do not discriminate the nest entrance from the foraging arena of the same colony. It indicates that outside the nest, considering only the methyl-branched alkanes, the marks are colony-specific but the nest entrance and the foraging arena are not well differentiated under our experimental conditions.

Discussion

Cuticular Profile of Lasius Niger The hydrocarbon profile of *L. niger* workers is composed of a set of linear alkanes and alkenes, and many mono-, di-, and trimethyl-alkanes. The most important peaks are mixtures of monomethyl- and dimethyl-alkanes. Little is known about *L. niger* hydro-

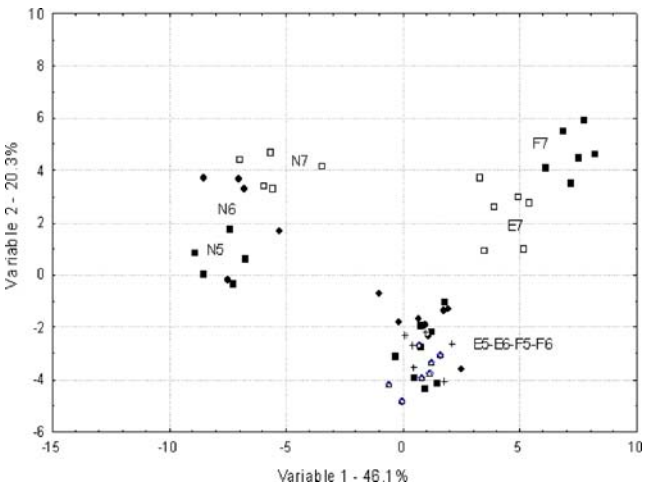

Fig. 5 Discriminant analysis of hydrocarbons obtained by SPME from inner walls of the nest (N), the entrance (E) and foraging arena (F) of colonies 5, 6, and 7. Lambda Wilk<0.001, F (240, 163)=2.92, P<0.001

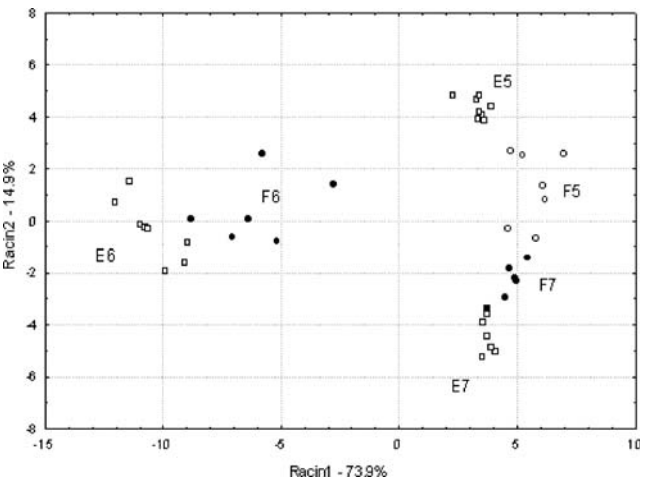

Fig. 6 Discriminant analysis of branched hydrocarbons obtained by SPME from the nest entrance (E) and foraging arena (F) of colonies T5, T6 and T7. Lambda Wilk<0.001, F (115, 73)=2.63, P<0.001. Distances not significantly different (NS): E6/F6 P=0.20; E5/F5 P=0.050; E7/F7 P=0.22 and F7/F5 P=0.23

carbons. Akino and Yamaoka described hydrocarbons of *L. niger* from Japan, which are completely different from European ones, but this species is now considered as a different twin species named *Lasius japonicus* (Akino and Yamaoka 2005). *L. niger* cuticular profiles appear to be stable in various areas in France, and also in Germany (Dinter et al. 2002), Belgium (A.L. unpublished) and Denmark (Dreier and D'Ettorre 2009). It also is the case for the invasive ant *Lasius neglectus*, which keeps the same profile in different locations (Ugelvig et al. 2008). There is no rule concerning the geographical variation of cuticular hydrocarbons in ants. Some species like *Cataglyphis cursor, C. iberica, Camponotus cruentatus*, or *Tetramorium* spp are highly variable (Nowbahari et al. 1990; Dahbi et al. 1996; Steiner et al. 2002; Boulay et al. 2007), while others like *Formica* do not change (Martin et al. 2008b).

Contrasting with the low geographical variation of chemical profiles, *L. niger* shows a strong local colony discrimination, as predicted for a monogynous and a little polyandric species [one to four copulations, see (Van der Have et al. 1988; Boomsma and Van der Have 1998; Aron et al. 2009)]. This seems to be general in monogynous species with independent founding like *Camponotus cruentatus* (Boulay et al. 2007).

The hydrocarbon quantity accounts for 0.1% of the head plus thorax mass, as is usual for ants (Lahav et al. 1998). The legs bear very small quantities of hydrocarbons that are colony specific but that have a slightly different profile from the body with an increase of linear alkanes, and tri - methyl-alkanes and alkenes in smaller proportions . Bagnères and Morgan (1991) also observed an increase in pentacosane (C25) on legs of *Myrmica rubra* queens.

Where do the leg hydrocarbons come from? Among the 20 glands described in ant legs, none seems to be involved in hydrocarbon production (Billen 2008), contrary to what was suggested by (Yamaoka and Akino 1994). Hence, hydrocarbons on the ant legs probably originate from self-grooming, but it is not clear how the difference in the profile can exist.

Are the Nest Walls Marked with Colony Odor? Nests or resting sites of many arthropods are marked and recognized by the owners. Several studies have confirmed such chemically-based nest recognition for solitary living arthropods such as tarantula spiders (Dor et al. 2008) or solitary bees (Guedot et al. 2006), for gregarious insects such as cockroaches (Ame et al. 2004) or triatomines (Lorenzo Figueiras and Lazzari 1998), and for social insects such as bumblebees (Saleh et al. 2007), honeybees (Butler et al. 1969) or ants (e.g., Depickère et al. 2004). In ants, volatile chemicals participate in nest recognition, for example in *Camponotus fellah* or *Pogonomyrmex badius* (see d'Ettorre and Lenoir 2009). Our results show for the first time that the inner walls inside the ant nest are marked with hydrocarbons comparable to those produced by the workers. This explains why many myrmecophiles obtain the colony odor by actively rubbing walls inside the nest (see reviews by Lenoir et al. 2001b; Akino 2008). Tricosane in particular is transmitted to myrmecophiles (Witte et al. 2008) agreeing with our results that show a high amount of linear alkanes inside the nest. Hydrocarbons found on the nest walls probably come from passive contacts with *L. niger* ant bodies. They also may be in the feces (Soroker and Hefetz 2000). Indeed, in the harvester ant *Messor capitatus*, the anal fluids deposited in the nest vicinity contain colony-specific cues that the ants use to recognize their nests from foraging areas (Grasso et al. 2005). We also established that the inside-nest odor is not completely colony-specific; for example, two colonies collected only a few meters apart had non discriminated odors.

The fact that ants have a colony specific cuticular odor and loose partially this specificity on the inside walls could be explained by the passive accumulation of compounds on the nest walls without permanent refreshing of the odor. Additionally, it has been observed that inside the nest, aliens generally are not rejected (Hölldobler and Wilson 1990). We thus hypothesize that the absence of alien rejection may be due not only to a higher threshold of discrimination by inner workers (Hölldobler and Wilson 1990) but also to an inside-nest colonial odor that is largely overlapping between colonies. We suggest that inside the nest, any social parasite will be considered as a fellow nestmate provided it has succeeded in "breaking the fortress" of the nest entrance, as stated by (D'Ettorre and Heinze 2001). This may explain why for instance in *Cardiocondyla elegans*, alate females are transported into alien nests by some workers, and are tolerated as soon as they have passed the nest entrance (Lenoir et al. 2006). Likewise, social parasites have a greater chance of being accepted once they succeed in entering the host nest. Such absence of alien rejection could be due to a saturation of the ant antennae receptors as hypothesized by (Ozaki et al. 2005). Our results show that the concentration of hydrocarbons actually is more important inside the nest than in the colony home-range, probably due to the high frequency of ant body contacts with the nest walls.

Are Nest Entrance and Home-Range Markings Colony Specific? Colony area marking is a widespread phenomenon in ants (see Hölldobler and Wilson 1990) and shows a diversity of glandular origin: postpharyngeal, metapleural, Dufour, cloacal glands, and also feces (Mayade et al. 1993; Cammaerts and Cammaerts 1998, 1999; Wenseleers et al. 2002; Grasso et al. 2005). In all cases, except two *Tetramorium* species (Cammaerts and Cammaerts 2000), area marking is colony-specific.

Our results on *L. niger* confirm previous behavioral results that show that the foraging area is chemically marked (Devigne and Detrain 2002, 2006; Depickère et al. 2004). It also confirms that marks consist of hydrocarbons identical to cuticular ones, as observed by (Yamaoka and Akino 1994). Beside ants, hydrocarbons left on the substrate are perceived by *L. niger* tended aphids and influence their dispersal (Oliver et al. 2007). In our experiments, the absence of active trail-laying behavior by *L. niger* indicates that marks are laid passively by walking ants, thus confirming previous observations (Depickère et al. 2004; Devigne and Detrain 2006). Footprint marks are colony-specific only when we consider the branched hydrocarbons in discriminant analyses. These footprints may act in conjunction with the trail pheromone as it was shown in the Asian species *L. japonicus* (Akino and Yamaoka 2005).

Even though the HC quantities on legs are small (less than 3 ng), the frequency of ants walking over an area may lead to a local density of marks high enough to be perceived by ants. It is well-known that social insects are highly sensitive to extremely low concentrations, for example, *Bombus* foragers rejecting flowers over which HCs doses of 10^{-12} ng/flower were deposited (Goulson et al. 2000). In *L. niger*, the perception of area marking promotes trail recruitment and food exploitation over familiar areas as compared to unexplored ones. The intensity of area marking is relevant for scouts to assess indirectly the colony occupancy at a location as well as the distance from the nest (Devigne and Detrain 2006). It is interesting to note that marks laid over the substrate have more alkanes than the cuticle, an increase in alkanes being also found on legs, which are in contact with the substrate. These higher concentrations of n-alkanes on the substrates may be due partly to differential melting points with branched hydrocarbons evaporating less rapidly than branched ones (Gibbs 1998; Hefetz 2007), and also to the fact that foragers have a larger proportion of straight-chain alkanes as compared to in-nest workers (Bonavita-Cougourdan et al. 1993; Greene and Gordon 2003). In *Formica exsecta* and *Pogonomyrmex barbatus*, this is due to exposure to high temperatures, UV, and low humidity (Wagner et al. 2001; Martin and Drijfhout 2009).

As regards the colony-specificity of area marking, similar chemical profiles are found at the nest entrance and in the foraging arena. This suggests that ants perceive the vicinity of their nest through an increased level of home-range marking rather than through qualitative changes in chemical marks. Volatile compounds also may be used by foragers to locate their nest entrance as has been shown recently on the desert ant *Cataglyphis fortis* (Steck et al. 2009).

Our main conclusion is that ants are faced with various blends of colony odors depending on their location. First, their own cuticular chemical profile varies from the head to the legs. Second, the odors of nestmates vary according to their caste (queen, workers, larvae, and males), their age, or their task performance. This indicates that, during ants' encounters, chemical cues used for nestmate discrimination are more complicated than a single bar-code. Finally, our chemical data confirm previous results that indicate that ants can perceive odors left by nestmates over substrates in their resting and foraging places (Devigne and Detrain 2006). Inside-nest odors are not colony-specific whereas area markings outside the nest are colony-specific, but at a low level. Even though discrimination may occur with branched alkanes, marks left at the nest entrance or on foraged areas are rich in linear alkanes that are considered to be poorly colony-specific.

Moreover, discrimination between colony home-range markings may be difficult to achieve due to the tiny amounts of footprint marks left over the substrate. As a consequence, in *L. niger*, defensive responses occur without a strict territorial marking but rather the ants rely on physical encounters to assess the relative force of opponent neighbors (Czechowski 1984). Area marking being poorly colony specific, rather acts as an indicator of the quality of a location. Conspecific cueing through area marking may influence the choice of a foraging space or of a nest site and thereby may promote a "shared information" strategy in *Lasius niger*. Based on the present work, one hopes that future studies will pay more attention to intracolonial sources of variability in odors, to the environmental influence on odors' colonial specificity, and to the sharing of conspecific chemical cues in insect societies.

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colony odor, and these compounds can be homogenized through all members of the colony during trophallaxis and allogrooming. In some ant species, individual ants admix their own CHC with those of nestmates in the postpharyngeal gland (Soroker et al. 1998). Polygynous ant species are expected to bear a gestalt label unique for each colony in a population, although extreme polygyny may limit the creation of unique labels and thereby reduce intercolony variation. The broad range of intracolony odors and lack of a distinct intrinsic colony odor may facilitate formation of unicolonial populations in which colony boundaries are weak or absent, although some odor differences arising from extrinsic factors may still exist (Hölldobler and Wilson 1990).

In the unicolonial, polygynous, and widespread invasive Argentine ant, *Linepithema humile*, CHC play a central role in nestmate recognition (Liang and Silverman 2000; Greene and Gordon 2007; Torres et al. 2007). An inverse relationship between intraspecific aggression and genetic similarity between nests in both native and introduced populations suggests that recognition cues are heritable (Tsutsui et al. 2000). Because prey-derived CHC can radically affect nestmate recognition and worker-worker aggression, it is clear that exogenous, environmentally-derived hydrocarbons also significantly influence the *L. humile* recognition system (Liang et al. 2001; Liang and Silverman 2000). Interestingly, the contribution of environmentally-derived cues to nestmate recognition varies among introduced populations, suggesting that genotypic diversity can affect the expression and perception of components of the *L. humile* recognition system (Buczkowski and Silverman 2006). In addition, *L. humile* colonies from the southeastern U.S. display varying levels of intraspecific aggression and greater genetic diversity than other introduced populations (Buczkowski et al. 2004). They also exhibit a greater range of interactions, varying from highly aggressive and lethal outcomes to colony fusion (Vásquez and Silverman 2008). Colony fusion is expected to affect nestmate discrimination by changing the genetic and phenotypic composition of the fused *L. humile* colony, and thereby offers a possible mechanism for the formation of large networks of interconnected, non-aggressive, multiple-queen nests, a social organization called "supercoloniality".

Supercolony formation in introduced *L. humile* populations may result not only from lower diversity of recognition cues, due to a loss of genetic diversity (Tsutsui et al. 2000), but also from selective mixing of non-nestmates that share higher levels of phenotypic similarity (Giraud et al. 2002). It has been shown that *L. humile* colonies in southeastern U.S. differ in ratios of worker CHC, and that colony fusion at 24 h correlates with similarities of worker CHC profiles (Vásquez and Silverman 2008). Because *L. humile* colonies are extremely polygynous, often containing thousands of queens, and the CHC profile of queens is qualitatively different from that of workers (Liang et al. 2001; de Biseau et al. 2004), we hypothesize that CHC similarity of queens is relevant to colony fusion. Consequently, we would expect a higher frequency of fusion between colony pairs that share greater similarity of their respective queen CHC. The disparate colony odors of workers and queens might together, or independently, guide the outcomes of behavioral interactions between colony pairs, leading to high levels of aggression if the CHC are relatively dissimilar, or to colony fusion if the CHC are relatively more similar. Moreover, fusion of colonies could promote exchanges of CHC within the worker and queen castes, resulting in changes in their respective CHC profiles. Such an exchange could result in blending of colony recognition cues, or the predominance of the chemical profile of one of the colonies. In either case, we suggest that changes in the CHC profile will facilitate colony integration. Therefore, we hypothesize that the chemical similarities of both worker and queen CHC profiles of aggressive *L. humile* colonies determine the probability of colony fusion; as colonies fuse, ants change their CHC profiles to a homogeneous chemical recognition odor. Moreover, behavioral interactions in *L. humile* vary according to the level of CHC similarity among workers (Suarez et al. 2002; Vásquez and Silverman 2008), and also to the degree of worker genetic similarity (Tsutsui et al. 2000; Vásquez and Silverman 2008). Thus, we further hypothesize that the level of genetic similarity between colony pairs also modulates colony fusion; we expect to find a relationship between overall genetic similarity and queen and worker CHC profile similarity between colonies.

To test these hypotheses, we conducted laboratory assays in which we paired experimental *L. humile* colonies, for which we had determined levels of overall genetic similarity and worker and queen CHC profile similarity. We also investigated whether between-colony similarity of the CHC profiles of queens and workers was associated with the frequency of colony fusion at 6 months, and with genetic similarity between colonies. We further compared the CHC profiles of queens and workers from colonies that fused or did not fuse after 6 months, to the profiles of the respective unpaired control colonies (i.e., no intercolony interactions) to determine if changes in worker and queen CHC profiles occurred as a result of fusion.

Methods and Materials

Experimental Colonies and Colony Fusion Assay Argentine ants were collected from five sites in the southeastern USA: Cary (CAR), Chapel Hill (CHH), Research Triangle Park (RTP), and Winston-Salem (FOR) in North Carolina; and

Greenville (COC) in South Carolina. Experimental colonies were established and maintained as previously described (Vásquez and Silverman 2008). Briefly, experimental colonies (5 queens, ca. 100 pieces of brood, 500 workers) were established 1–2 mo after collection in individual Fluon-coated trays (17×25×11 cm), and provided an artificial nest, 25% sucrose solution, artificial diet, and a water source. All queens and 50 workers from each colony were marked with water-based paint (colony 1 = pink; colony 2 = yellow) to observe mixing of individuals and fusion events. Trays were connected through a vinyl tube (12 cm long) that was unblocked after a 24 h acclimation period. Controls consisted of experimental colonies that were not paired to any foreign colony. We recorded total number of workers fighting, and total number of dead workers in each tray for all ten pairwise colony combinations each hour for 6 h, and 24 h after colonies were allowed to interact. Colony pairs were inspected for fusion daily, from day 2 to day 30, and monthly from month 2 to month 6. Fusion was defined as the presence of queens from both colonies and all brood in the same nest (Vásquez and Silverman 2008).

Extraction, Isolation, and Chemical Analysis of Cuticular Hydrocarbons Six months after the colony fusion assay started, we collected queens (4–11) and workers (80–100) from the following colony pairs: CAR-CHH, CAR-COC, CAR-RTP, CHH-FOR, CHH-RTP. Ants were placed individually (queens) or in groups of ten (workers) in glass vials and stored at −20°C for subsequent CHC analysis. Queens were matched to their colony of origin based on the water-based paint mark. We also collected and stored queens (6–10) and workers (60) from each of the five unpaired control colonies. The CHC profiles of queens and workers from unpaired control colonies were analyzed to determine if colonies could be distinguished based on CHC, and to establish any relationship between CHC similarities between colonies and colony fusion at 6 months. We also compared queen and worker CHC profiles from colony pairs with those of their respective unpaired control colonies to determine if changes in CHC occurred after colony fusion. Cuticular lipids of thawed queens and workers were extracted, and CHC purified on silica gel as previously described (Vásquez and Silverman 2008). Capillary gas chromatography (GC) was carried out using an HP5890 gas chromatograph equipped with a DB-5 column (30 m×0.25 mm×0.5 µm film thickness). Extracts were introduced into a split-splitless inlet operated at 300°C in splitless mode (2 min purge) with helium as carrier gas at a linear velocity of 30 cm sec^{-1}. Oven temperature was held at 80°C for 2 min, increased to 270°C at 20°C min^{-1}, then to 310°C at 3°C min^{-1}, and held for 20 min. Individual queen extracts were resuspended in 20 µl hexane and 0.5 µl (0.025 queen equivalents) analyzed; extracts of ten workers were resuspended in 10 µl hexane, and 2 µl (2 worker equivalents) were injected. Peaks were integrated, and their individual percentages of the total CHC peak area calculated; only peaks with a mean percent area across all colonies of ≥1% were used in data analysis. The identity of discriminating peaks was determined by gas chromatography-mass spectrometry (GC-MS) and by comparing their retention times with those of alkane standards (*n*-C23–*n*-C38) (Liang et al. 2001; de Biseau et al. 2004). GC-MS analyses of CHC were performed on an HP6890 GC, equipped with an HP-5MS column (30 m×0.25 mm×0.25 µm film thickness), connected to an HP5973A mass selective detector. The inlet was operated at 300°C in splitless mode with helium as carrier gas at a linear velocity of 45 cm sec^{-1} (2 min purge). Data were recorded in scan mode (25–550 *m/z)* using electron impact ionization.

Genetic Similarity Among Colonies Genetic similarity among colonies used in the fusion assay was assessed by using microsatellite markers. DNA was extracted from 15 workers from each of the experimental colonies (CAR, CHH, COC, FOR, and RTP), and 7–17 queens from CAR, CHH, and RTP, using the DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) and analyzed at eight microsatellite loci: Lhum-11, Lhum-13, Lhum-19, Lhum-28, Lhum-35, Lhum-39 (Krieger and Keller 1999), Lihu-M1 and Lihu-T1 (Tsutsui et al. 2000). PCR reactions were performed as previously described (Buczkowski et al. 2004). Products were separated on 6.5% KB^{Plus} polyacrylamide sequencing gels with a 4300 LI-COR DNA analyzer. Microsatellite alleles were scored using GeneImagIR software (Scanalytics Inc., Billerica, MA, USA). Levels of genetic similarity among colonies were estimated based on the percentage of alleles shared between groups (Tsutsui et al. 2000). Genetic differentiation (F_{ST}) among Argentine ants from different locations was estimated with the program FSTAT v.2.9.3.2 (Goudet 1995).

Statistical Analyses Data analyses were performed with SAS 9.1.3 statistical software (SAS 2004). We performed a stepwise discriminant analysis (stepwise DA) on the transformed quantitative CHC data of control colonies by using PROC STEPDISC to identify variables (GC peaks) that differed significantly between groups of queens or workers. Peak areas were transformed following Aitchison's formula: $Z_{ij}=\ln[Y_{ij}/g(Y_j)]$, where Z_{ij} is the standardized peak area *i* for individual *j*, Y_{ij} is the peak area *i* for individual *j*, and $g(Y_j)$ is the geometric mean of all peaks for individual *j* (Reyment 1989). Only transformed variables that met the assumption of homogeneity of variance (Brown and Forsythe's test) were used in stepwise DA. We then performed a canonical DA on the selected peaks using PROC DISCRIM. Pairwise generalized square distances between group means (cent-

roids) were used as an estimate of the degree of CHC profile differentiation between colonies. To determine changes in queen and worker CHC profiles, of fused and non-fused colonies vs. their respective controls, we first estimated the linear discriminant function coefficients only for unpaired control colonies by using PROC DISCRIM, and then computed the linear discriminant function for fused and non-fused colony pairs using these coefficients.

Spearman rank correlation coefficients were used to determine relationships among percentage of colonies fused at 6 months and genetic similarity vs. queen and worker CHC similarity. The significance of the correlation coefficient was tested by Mantel correlation test in GENEPOP using 10,000 permutations. All means reported are followed by standard errors (SEM).

Results

Colony Fusion Assay The percentage of colony fusion at 6 months varied across *L. humile* colony pairs; all CAR-RTP, CHH-RTP, and COC-FOR replicates fused, none of the CAR-COC, CHH-COC, CHH-FOR, and COC-RTP replicates fused, and 40% of the CAR-FOR and FOR-RTP replicates, and 20% of the CAR-CHH replicates, fused. All queens from one of the paired colonies in replicates that did not fuse were killed. Interestingly, in CHH-FOR, unlike for all other non-fused colony pairs, we observed workers from both colonies mixing in all replicates; all CHH queens were killed by FOR workers.

Chemical Analysis of Cuticular Hydrocarbons: Cuticular Hydrocarbon Patterns of L. humile Unpaired Control Colonies GC and GC-MS analyses of queen and worker CHC showed that chemical profiles differed quantitatively and qualitatively between the castes (Fig. 1). Thirty one CHC peaks for queens and 44 CHC peaks for workers were consistently found in each colony; 21 compounds were common to both castes (Fig. 1). The stepwise DA performed on 25 queen CHC peaks that met the assumption of homogeneity of variance selected nine peaks that distinguished queens from different colonies (*Wilks'* $\lambda<0.01$, $F=13.63$, $df=36, 125.4$, $P<0.001$) (Fig. 2a), and had generalized square distances between colony centroids (i.e., chemical distance) ranging from 7.5 (CAR-RTP) to 123.2 (CAR-COC). All queen peaks that differed among colonies included the same discriminating compounds previously identified in Argentine ant queen adoption assays (Vásquez et al. 2008) as well as nonacosene (xC29:1) and *n*-hentriacontane (*n*-C31). The stepwise DA performed on 34 worker CHC peaks selected 10 peaks that grouped all workers from control colonies according to their colony of

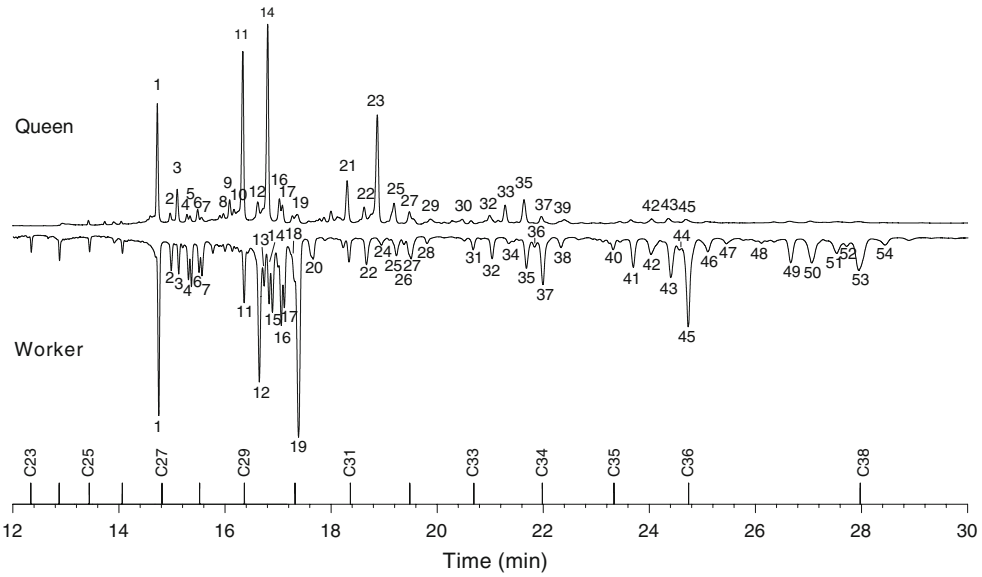

Fig. 1 Gas chromatogram of cuticular hydrocarbon profiles of *Linepithema humile* queen and worker. **1:** *n*-C27; **2:** UI; **3:** 5-MeC27; **4:** 11- and 13-MeC27; **5:** 3-MeC27; **6:** *n*-C28; **7:** UI; **8:** UI; 9: xC29:1; **10:** UI; **11**: *n*-C29; **12:** 11- and 13- and 15-MeC29; **13:** UI; **14:** 5-MeC29; **15:** UI; **16:** 3-MeC29; **17:** 5-MeC30; **18:** UI; **19:** *n*-C30; **20:** UI; **21:** *n*-C31; **22:** 11- and 13- and 15-Me C31; 23: 5-MeC31; 24: UI; **25:** x,y-diMeC31; 26: 3-MeC31; **27:** x,y,z-triMeC31; 28: UI; **29:** UI; **30:** xC33:1; 31:*n*-C33; **32:** 13- and 15- and 17-MeC33; **33:** 5-MeC33; 34: UI; **35:** 5-MeC34; 36: 5- and 15- and 19-triMeC33; **37:** UI; **38:** UI; 39: UI; **40:** UI; 41: 13- and 15- and 17-MeC35; **42:** 15,19-diMeC35; **43:** 5,15- and 5,17-diMeC35; 44: UI; **45:** 5,13,17- and 5,15,19-triMeC35; **46:** 3,13,17- and 3,15,17-triMeC35; **47:**UI; **48:** 13- and 15- and 17- and 19-MeC37; **49:** 15,19-diMeC37; **50:** 5,15- and 5,17-diMeC37; **51:** 5,15,19- and 5,13,17-triMeC37; **52:**UI; **53:**UI; **54:** UI. Numbers in *bold* indicate peaks used in stepwise discriminant analyses. Alkane standards are shown on the x-axis. UI: unidentified compound

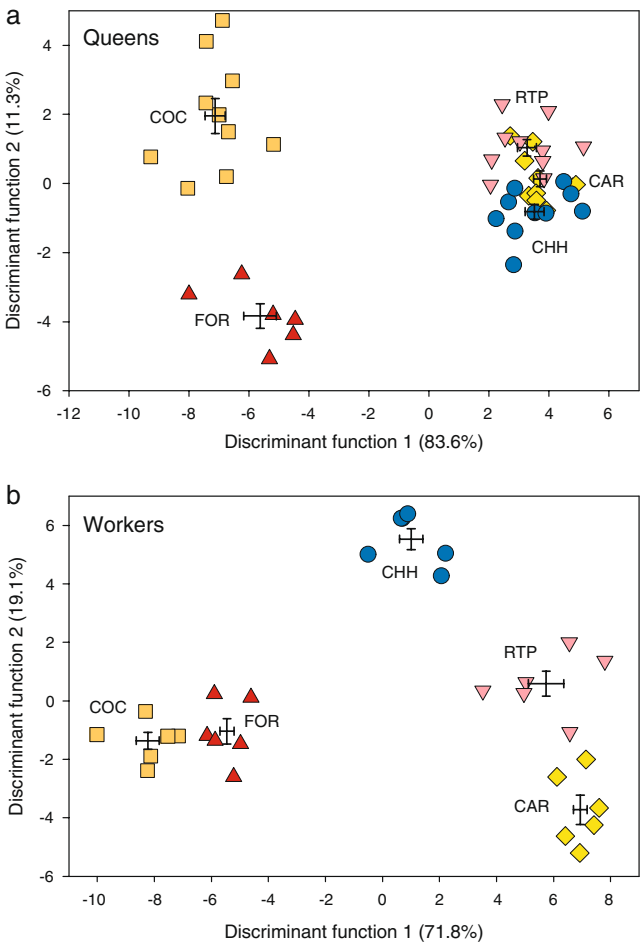

Fig. 2 Discriminant analysis of *Linepithema humile* cuticular hydrocarbons (CHC). **a** Discriminant analysis based on nine variables (CHC peaks) selected by stepwise discriminant analysis for queens from five *L. humile* colonies (CAR, CHH, COC, FOR, RTP). **b** Discriminant analysis based on ten variables (CHC peaks) selected by stepwise discriminant analysis for workers from five *L. humile* colonies (CAR, CHH, COC, FOR, RTP). *Bars* represent standard errors for a colony mean (centroid)

origin (*Wilks'* $\lambda<0.01$, $F=13.54$, $df=40, 62.5$, $P<0.001$) (Fig. 2b), with generalized square distances between colony centroids ranging from 24.8 (CAR-RTP) to 243.2 (CAR-COC). Five of the discriminating worker peaks were identified as 11- and 13-methylheptacosane (11- and 13-MeC27), 3-methylnonacosane (3-MeC29), 11- and 13- and 15-methylhentriacontane (11-, 13-, and 15-MeC31), 5,13,17- and 5,15,19-trimethylpentatriacontane (5,13,17- and 5,15,19-triMeC35), and 5,15- and 5,17-dimethylheptatriacontane (5,15- and 5,17-diMeC37).

Chemical Analysis of Cuticular Hydrocarbons: Cuticular Hydrocarbon Patterns of Fused and Non-fused L. humile Colonies To investigate the effects of colony fusion on queen CHC profiles, we computed discriminant functions for each colony pair by using discriminant function coefficients estimated from nine discriminating peaks derived from the control colonies. The CHC profiles of queens in colony pairs that fused (CAR-CHH, CAR-RTP, and CHH-RTP) occupied a much broader DA space and were spread across the two control groups (Fig. 3), indicating that queens in fused colonies formed a group with higher CHC variability that generally could not be distinguished unequivocally accord-

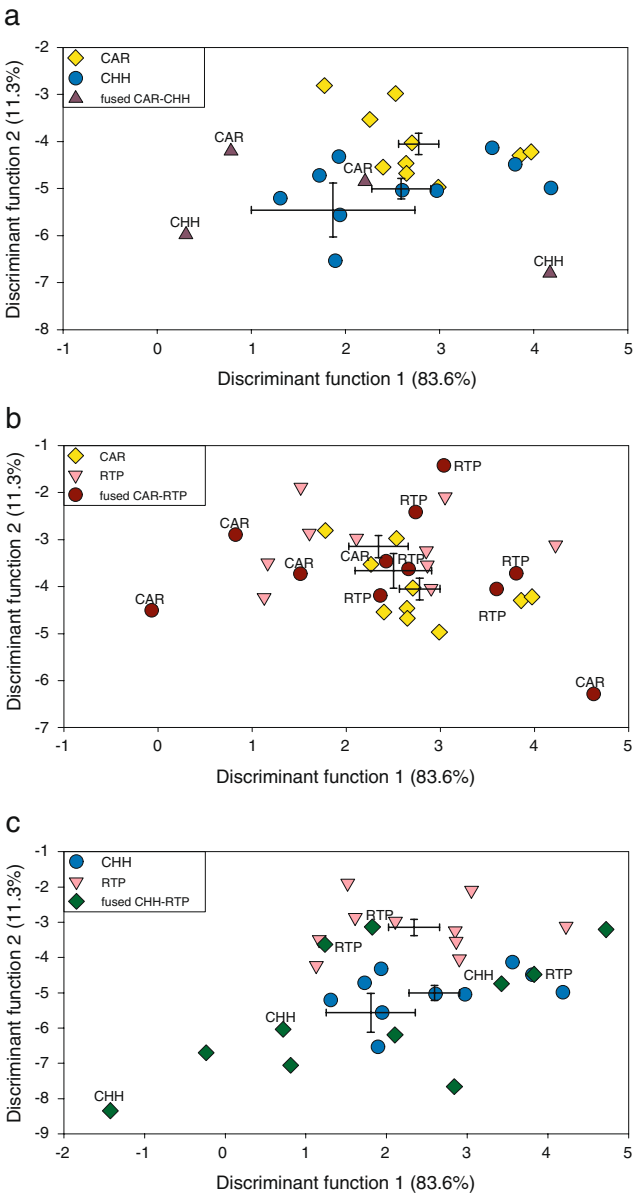

Fig. 3 Linear discriminant functions for cuticular hydrocarbons of *Linepithema humile* queens of fused colony pairs. **a** CAR-CHH (centroid: X=1.87±0.87, Y=−5.46±0.58) and unpaired controls CAR and CHH. **b** CAR-RTP (centroid: X=2.50±0.41, Y=−3.67±0.37) and unpaired controls CAR and RTP. **c** CHH-RTP (centroid: X=1.80±0.55, Y=−5.56±0.55) and unpaired controls CHH and RTP. Functions plotted based on nine variables. Bars represent standard errors for a colony mean (centroid). CAR centroid: X=2.78±0.21, Y=−4.05±0.23; CHH centroid: X=2.59±0.31, Y=−5.01±0.22; RTP centroid: X=2.34±0.32, Y=−3.15±0.24

ing to their colony of origin. This was especially apparent for the CHC profiles of five CAR queens and six RTP queens from CAR-RTP colonies that fused (Fig. 3b). The CHC profiles of two CAR and two CHH queens in fused CAR-CHH colonies made up a group with higher variability than their respective controls, but appeared more similar to CHH control queens (Fig. 3a), suggesting that CAR queens may have acquired CHH hydrocarbons. In the CHH-RTP pairings, six queens of known identity had similar CHC profiles to their controls, CHH and RTP, yet displayed higher CHC variability (Fig. 3c).

In colony pairs that did not fuse (CAR-COC and CHH-FOR), the CHC profiles of queens were more similar to the CHC profile of their respective unpaired control queens, with no overlap with the CHC of the other colony's queens (Fig. 4). In CAR-COC, the CHC profiles of the seven CAR queens and three COC queens examined were more similar to their respective unpaired controls than to those of queens of the other colony, and in CHH-FOR, the CHC profiles of ten queens known to be FOR were similar to those of the FOR control group (Fig. 4b).

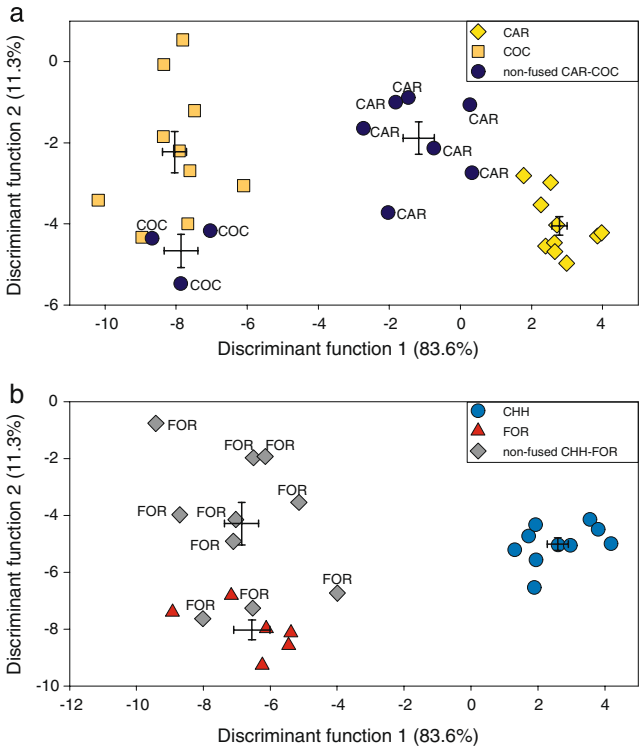

Fig. 4 Linear discriminant functions for cuticular hydrocarbons of *Linepithema humile* queens of non-fused colony pairs. **a** CAR-COC (CAR centroid: X=−1.18±0.44, Y=−1.89±0.40; COC centroid: X=−7.86±0.47, Y=−4.67±0.41) and unpaired controls CAR and COC. **b** CHH-FOR (FOR centroid: X=−6.86±0.51, Y=−4.28±0.75) and unpaired controls CHH and FOR. Functions plotted based on nine variables. *Bars* represent standard errors for a colony mean (centroid). CAR centroid: X=2.78±0.21, Y=−4.05±0.23; CHH centroid: X=2.59±0.31, Y=−5.01±0.22; COC centroid: X=−8.04±0.34, Y=−2.23±0.51; FOR centroid: X=−6.55±0.54, Y=−8.02±0.35

When we plotted worker CHC profiles of each colony pair with profiles of their respective unpaired controls, using ten discriminating peaks, we found that CHC profiles in colony pairs that fused (CAR-CHH, CAR-RTP, and CHH-RTP) occupied a much broader and relatively intermediate DA space between the unpaired control colonies, CAR, CHH, and RTP (Fig. 5). Unlike for the queens, the worker CHC profiles were not scattered throughout the control CHC profiles, probably because each sample consisted of ten randomly sampled workers of unknown colony affiliation. However, we presume that the average CHC profile of workers in fused colonies reflected a homogenized CHC composition of both colonies. In the non-fused CAR-COC pair, samples were taken from replicates that were either CAR or COC, based on colony queen identity, with CHC profiles of workers similar to CHC profiles of the workers of the respective controls (Fig. 6a). However, since CAR-COC workers were not marked, we could not rule out the possibility that these samples may have included workers from both colonies. Interestingly, in CHH-FOR pairings, all CHH queens were killed, but CHH and FOR workers apparently mixed. The CHH-FOR workers had a broad and largely distinctive CHC profile that, for some groups, was more similar to FOR workers than to CHH workers (Fig. 6b).

Genetic Similarity Among Colonies Genetic similarity among colonies (percent alleles shared) in the fusion assay varied across colony pairs, ranging from 35.3% (CHH-COC) to 74.3% (CAR-RTP) (Table 1). The overall genetic differentiation among colonies (F_{ST}) was, on average, 0.201±0.055 with pairings estimates (pairwise F_{ST}) ranging from 0.051±0.015 (CHH-RTP) to 0.431±0.090 (CHH-COC) (Table 1).

Correlations Among Cuticular Hydrocarbon Similarity, Genetic Similarity, and Colony Fusion We found a significant relationship between CHC similarities (i.e., low CHC distance) of queens from control colonies (Table 1) and the frequency of colony fusion at 6 months (*Mantel test* P=0.032) (Fig. 7). A similar relationship was evident between control worker CHC similarities (Table 1) and colony fusion at 6 months (*Mantel test* P=0.050) (Fig. 7). We found a strong relationship between percent alleles shared and queen and worker CHC profile similarity between colonies (*Mantel tests* P=0.007, and P=0.009, respectively) (Fig. 8a). Similarly, we found a relationship between pairwise F_{ST} and queen and worker CHC profile similarity between colonies (*Mantel tests* P=0.023, and P=0.040, respectively) (Fig. 8b). Also, we found a relationship between colony fusion at 6 months and percent alleles shared (*Spearman's* r=0.666, *Mantel test* P=0.042) and pairwise F_{ST} (*Spearman's* r=−0.822, *Mantel test* P=0.009).

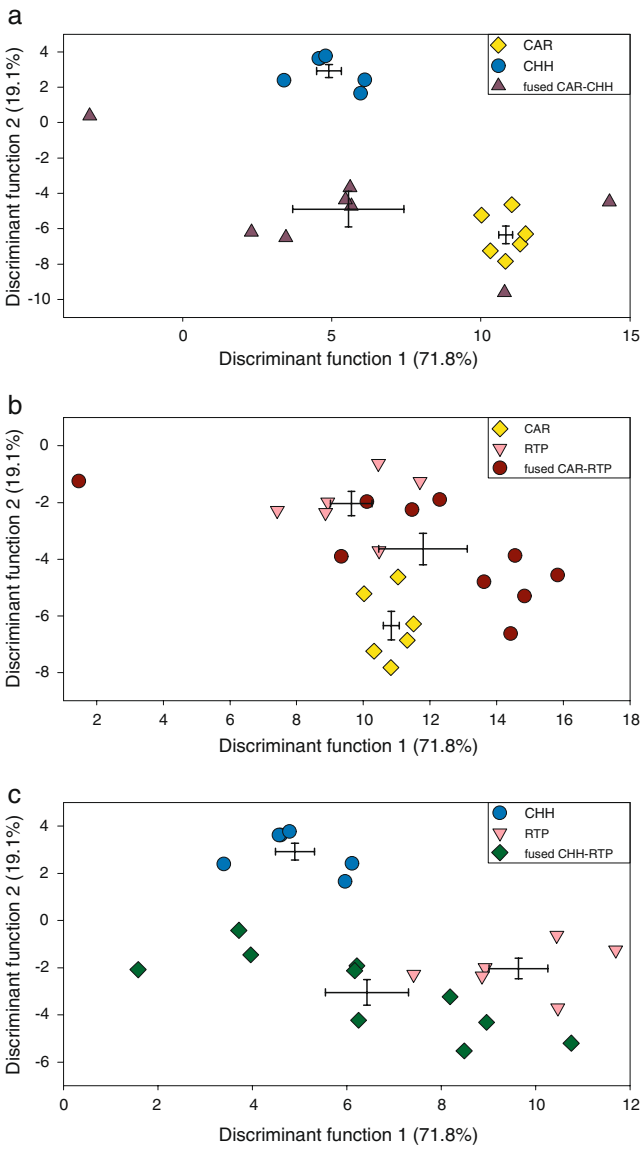

Fig. 5 Linear discriminant functions for cuticular hydrocarbons of *Linepithema humile* workers of fused colony pairs. **a** CAR-CHH (centroid: X=5.56±1.86, Y=−4.89±1.00) and unpaired controls CAR and CHH. **b** CAR-RTP (centroid: X=11.79±1.33, Y=−3.64±0.55) and unpaired controls CAR and RTP. **c** CHH-RTP (centroid: X=6.43±0.88, Y=−3.05±0.54) and unpaired controls CHH and RTP. Functions plotted based on 10 variables. *Bars* represent standard errors for a colony mean (centroid). CAR centroid: X−10.84±0.24, Y=−6.35±0.50; CHH centroid: X=4.90±0.41, Y=2.91±0.36; RTP centroid: X=9.64±0.62, Y=−2.03±0.43

Discussion

We demonstrated, for both queens and workers of *L. humile*, that similarity of CHC profiles between unrelated colonies was positively associated with colony fusion, and that the CHC profiles of fused colonies tended to be composed of a blend of CHC from both colonies. Our findings indicate that merging of unrelated *L. humile* colonies can lead to changes in the composition of recognition cues among members of the fused colonies, and possibly to expansion of the recognition template. This likely explains our earlier observation that fused colony pairs directed less aggression toward both source colonies compared to colonies that did not fuse (Vásquez and Silverman 2008). Discrimination abilities are important in structuring *L. humile* societies (Tsutsui et al. 2000; Giraud et al. 2002), and our results suggest that colony fusion can lead to more open colonies that may accept certain non-nestmates, further supporting the idea that expansive colonies in the introduced range of *L. humile* result from mixing or fusion of unrelated colonies.

Linepithema humile workers and queens differ considerably in their CHC profiles; workers have substantial amounts of dimethyl- and trimethylalkanes (diMe- and triMeC33, -C35 and -C37), whereas queens lack (or have very low amounts of) these compounds, but have monomethylalkanes (5-MeC27 to 5-MeC34) and alkenes (C29:1, C31:1, C33:1) as major components (Liang et al. 2001; de

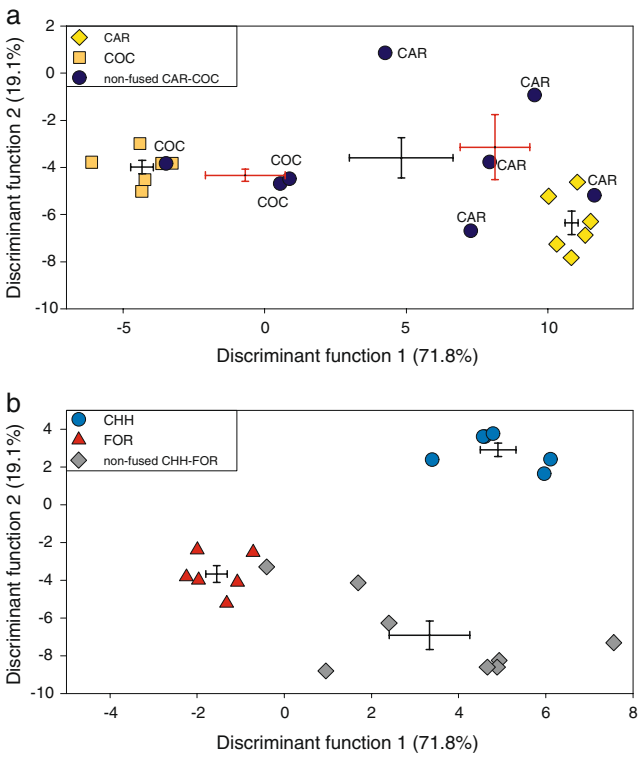

Fig. 6 Linear discriminant functions for cuticular hydrocarbons of *Linepithema humile* workers of non-fused colony pairs. **a** CAR-COC (CAR centroid: X=8.13±1.23, Y=−3.15±1.38; COC centroid: X=−0.69±1.40, Y=−4.33±0.26; or CAR-COC centroid: X=4.82±1.83, Y=−3.59±0.86) and unpaired controls CAR and COC. **b** CHH-FOR (centroid: X=3.34±0.92, Y=−6.90±0.76) and unpaired controls CHH and FOR. Functions plotted based on 10 variables. Bars represent standard errors for a colony mean (centroid). CAR centroid: X=10.84±0.24, Y=−6.35±0.50; CHH centroid: X=4.90±0.41, Y=2.91±0.36; COC centroid: X=−4.33±0.40, Y=−3.99±0.28; FOR centroid: X=−1.55±0.25, Y=−3.67±0.44

Table 1 Chemical distances and genetic similarity among *Linepithema humile* colonies. Generalized squared distances between colony centroids (i.e., chemical distance), genetic similarity (% alleles shared), and genetic differentiation (Pairwise F_{ST}) between colonies of a *L. humile* fusion assay. Chemical distances were calculated by discriminant analysis of the cuticular hydrocarbons of workers and queens from control (unpaired) colonies

Colony pair	Workers 10 variables	Queens 9 variables	Alleles shared (%)	Pairwise F_{ST}
CAR-CHH	120.96	8.52	64.50	0.160±0.046
CAR-COC	243.19	123.19	43.80	0.312±0.053
CAR-FOR	174.85	105.36	57.58	0.212±0.055
CAR-RTP	24.83	7.50	74.29	0.065±0.022
CHH-COC	140.56	122.32	35.29	0.431±0.090
CHH-FOR	98.24	96.46	64.50	0.255±0.128
CHH-RTP	51.06	8.09	66.70	0.051±0.015
COC-FOR	46.71	36.64	62.07	0.212±0.038
COC-RTP	205.91	111.14	40.00	0.312±0.115
FOR-RTP	144.86	103.63	60.53	0.200±0.125

Biseau et al. 2004). In this study, we found that quantitative differences of worker and queen CHC profiles reflect colony identity, and that a statistically derived subset of compounds may mediate colony discrimination. Worker CHC supplementation studies (Greene and Gordon 2007; Torres et al. 2007) suggest that a mixture of CHC of different structural classes, with varying ratios across colonies rather than a few compounds from a single structural class, may be used as nestmate recognition cues in *L. humile*. Our findings support this view and, in addition, suggest that alkenes and monomethylalkanes may be important in queen discrimination, while dimethyl- and trimethylalkanes and other unidentified long-chain CHC may be important in worker recognition. Methyl-branched alkanes, *n*-alkanes, and an alkene/*n*-alkane mixture have been shown to be important colony recognition cues in wasps (Dani et al. 1996; Gamboa et al. 1996). In ants, methyl-branched CHC are more colony-specific than *n*-alkanes (Astruc et al. 2001), although

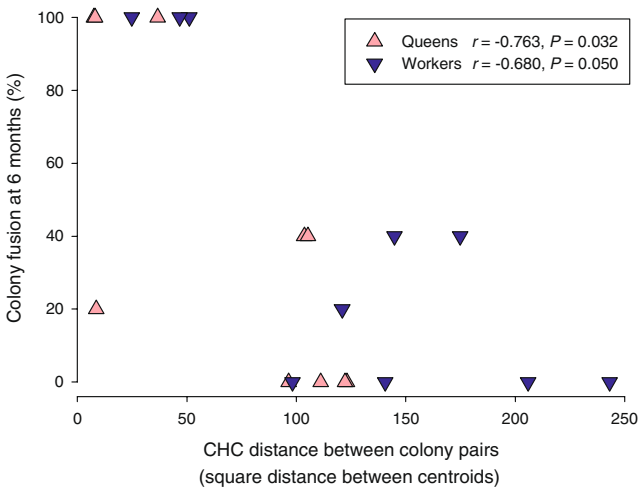

Fig. 7 Relationship (Spearman's *r*) between *Linepithema humile* colony fusion at 6 months and queen and worker cuticular hydrocarbon profile similarity based on nine and ten transformed variables, respectively

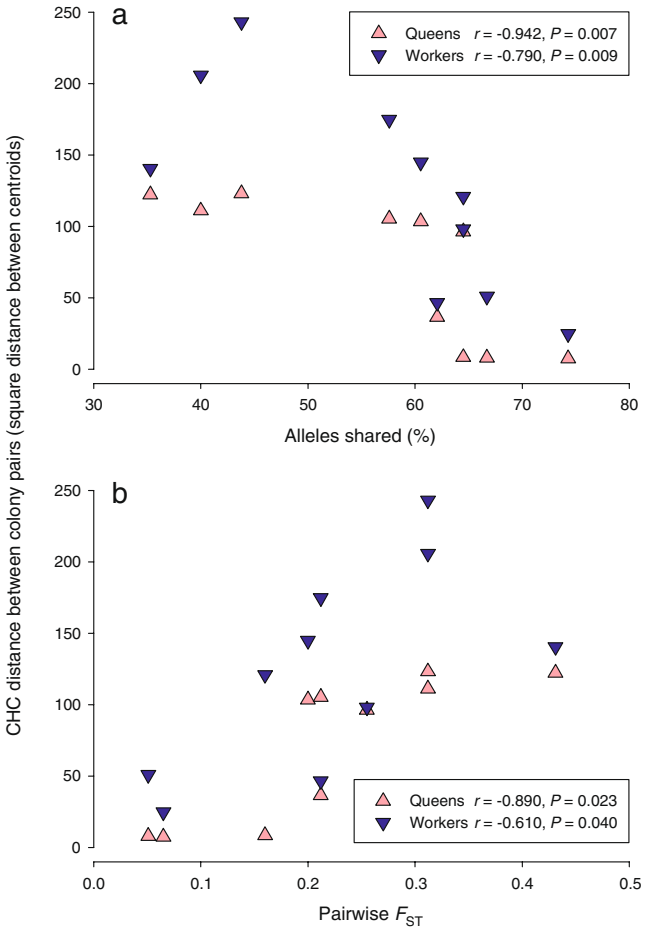

Fig. 8 Relationship (Spearman's *r*) between *Linepithema humile* cuticular hydrocarbon similarity and genetic similarity. **a** Genetic similarity (% alleles shared) between colonies versus queen and worker cuticular hydrocarbon similarity (generalized square distance between colony centroids). **b** Pairwise F_{ST} between colonies versus queen and worker cuticular hydrocarbon similarity (generalized square distance between colony centroids)

dimethylalkanes seem not to be important in nestmate recognition in *Cataglyphis* species (Dahbi et al. 1996). Chemical supplementation studies, testing the various alkanes and alkenes identified in our study, should be conducted to test the behavioral role of these compounds.

The distinct CHC profiles of queens and workers and our statistical identification of different CHC that distinguish queens and workers from different colonies suggest that *L. humile* does not form a unique colony odor distributed among all colony members (castes). Instead, individuals may have either a reproductive or sterile worker odor, that together constitutes a more complex colony template. A similar mechanism of template formation has been suggested for mixed-species groups in which individuals seem to learn and memorize allospecific cues early in adult life (Errard 1994). Two other possibilities are, that individuals bear their own endogenous cues and that these are matched with a learned Gestalt-type template, or that CHC, common to both queens and workers, are used as a single colony recognition template. In *Camponotus vagus*, dimethylalkanes are present across all castes, and are thought to be colony chemical cues, while *n*-alkanes and monomethylalkanes characterize larvae, workers, sexuals, and queens (Bonavita-Cougourdan et al. 1993), and may represent caste-specific cues.

In *L. humile*, genetically-based recognition cues appear to play a major role in nestmate discrimination among genetically diverse populations, whereas environmentally-derived CHC appear to be important in *L. humile* worker recognition in populations with reduced genetic variability (Buczkowski and Silverman 2006). In our study, CHC profiles of workers and queens are likely intrinsic because colonies were exposed to identical environmental conditions, and individuals were sampled 6 months after the start of the experiment. This explains the stronger association we found between worker CHC similarity and colony fusion at 6 months than the one found for colony fusion at 24 h (Vásquez and Silverman 2008). In the latter study, workers may have possessed both exogenous and endogenously-derived CHC. Temporal variation in worker CHC may also explain fusion events that occurred several weeks after interactions started, although factors (e.g., colony phenology, caste ratios, worker age) other than recognition cue phenotypic similarity could also govern the outcome of group interactions. In some colony pairs, CHC profiles between queens were more similar than those between workers, suggesting that workers may not be aggressive toward foreign queens but they may be aggressive toward workers from the foreign colony. Therefore, the outcome of group interactions may not exclusively reflect worker discrimination capability, or individual worker interests, but that of the whole group.

CHC profiles of queens changed after colony fusion, with the CHC profile of queens, as a group, in the fused colony not resembling the CHC profiles of either parent colony or indeed of a hybrid intermediate. Instead, profiles of queens varied across the range of profiles of both source colonies. This, together with the observation that queens could be assigned to their colony of origin based on their CHC profiles in non-fused colony pairs, suggests that by exchanging CHC, queens may match phenotypes in both colonies, thereby forming a broader queen recognition template. The collective worker CHC composition found in fused colony pairs suggests that mixing of worker CHC between colonies may have occurred. Transfer of CHC among individuals of the same colony, among mixed species, and in dulotic and inquiline species, has been well documented (Howard et al. 1980; Soroker et al. 1994). Cue exchange within castes could have occurred through direct body contact, grooming, and trophallaxis, in the same way that interactions with adult workers allow newly eclosed ant workers (callows) to acquire a colony's odor (Vander Meer and Morel 1998), or interactions with heterospecifics result in mixed hydrocarbon profiles in ants (Errard et al. 2006).

Colony fusion between aggressive *L. humile* colonies can also be predicted from the genetic similarity between colonies. This is in line with studies showing that aggression levels between colonies, or populations, of social insects are based on similarity of genetically-based CHC profiles (Dronnet et al. 2006). In *L. humile*, intraspecific aggression is based on levels of genetic similarity (Tsutsui et al. 2000), and is also guided by worker and queen hydrocarbons (Greene and Gordon 2007; Torres et al. 2007; Vásquez et al. 2008). However, the roles of chemical and genetic factors in regulating behavioral interactions among conspecifics have not been tested simultaneously. By combining behavioral, chemical, and genetic approaches, we demonstrated that both genetic and chemical factors play important roles in nestmate recognition and in shaping colony phenotypic composition, thereby offering a potential mechanism for changes in social structure in the introduced range.

While both queen and worker CHC similarity between colonies can guide fusion between genetically distinct *L. humile* colonies, changes in CHC patterns of colonies that had fused suggest homogenization of colony CHC between fused colony pairs, thus explaining reduced aggression toward unpaired control colonies (Vásquez and Silverman 2008) and fused colony cohesion. High aggression between non-fused colony pairs (winning colony) and their respective unpaired controls (defeated colony) after 6 months may be explained by maintenance of the colony chemical signatures. In line with previous studies that found an association between worker CHC similarity and intraspecific aggression in field and laboratory *L. humile* colonies (Liang and Silverman 2000; Suarez et al. 2002; Buczkowski and Silverman 2006), we found that worker and queen CHC

profile similarity is associated with fusion of unrelated colony pairs. Variation in colony genotypic composition, through mixed workers and queens, may lead to the formation of a new colony odor, implying that an updated recognition template must also be learned. It has been proposed that the greater the dissimilarity in CHC profiles between ant species that mix, the lower the aggression toward other ant species, due to a broader template (Errard et al. 2006). Similarly, increased phenotypic cue diversity in fused colonies should result in a much broader template. This may have implications at the population level, since changes in social structure may arise from changes in recognition cue diversity and/or template formation. Therefore, by increasing colony phenotypic diversity, fusion between unrelated colonies may be a proximate mechanism involved in the formation of expansive *L. humile* supercolonies in the introduced range.

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et al. 2005). Carboxylic acids also play a crucial role in the behavior of *Ae. aegypti* (Bosch et al. 2000) together with ammonia and lactic acid (Geier et al. 1999). Humans have uniquely high levels of skin-borne lactic acid compared to other mammals and chickens. Therefore, lactic acid may play a critical role in the host-seeking behavior of anthropophilic mosquitoes, which seem to have adapted to utilize this human compound (Dekker et al. 2002). By contrast, ammonia is a typical animal excretory product found on the skin of all vertebrates. Skin bacteria convert human skin lipids into unusual long chain carboxylic acids, whereas long chain carboxylic acids can be converted into volatile carboxylic acids (C2–C14). In addition, short chain carboxylic acids (C2–C5) can be derived from glycerol, amino acids, and lactic acid (James et al. 2004).

Although aliphatic carboxylic acids are involved in the attraction of *An. gambiae s.s.* to humans, the role of the various acids remains elusive. Therefore, we aimed to examine the role of individual aliphatic carboxylic acids using synthetic blends. The carboxylic acids were tested individually (in combination with ammonia + lactic acid), and those that augmented the attractive effect were subsequently tested in combinations, using two different bioassay systems. Both optimization and subtraction experiments were performed in order to examine the contribution of individual carboxylic acids to a multi-component blend.

Methods and Materials

Mosquitoes The colony of *Anopheles gambiae sensu stricto* (hereafter *An. gambiae*) was maintained at Wageningen University, The Netherlands, and originally obtained from Suakoko, Liberia (courtesy Prof. M. Coluzzi, Rome, Italy). The mosquitoes have been cultured in the laboratory since 1988 with blood meals from a human arm twice a week. The mosquito colony was kept in a climate room at 27±1°C, 80±5% RH and a photo-scotophase of 12/12 hL/D. Adult mosquitoes were maintained in 30×30×30 cm gauze cages with access to a 6% glucose solution on filter paper. Larvae were reared on tap water in plastic trays and fed daily with Tetramin® baby fish food (Melle, Germany). Pupae were collected daily and placed into adult cages for emergence.

Olfactometer A dual-port olfactometer, consisting of a flight chamber (1.60 x 0.66×0.43 m) with glass walls and a Luxan top, was used to study the behavioral responses of female mosquitoes to different odor stimuli. Pressurized air was charcoal filtered, humidified, and led through two Perspex mosquito trapping devices connected to two ports (4 cm diam and 28 cm apart) in the flight chamber (flow rate of 20.6±1.4 cm/s). The light from one tungsten light bulb (75 Watt) was filtered and scattered through a screen of yellow cloth hanging ± 1 m above the flight chamber. This resulted in dim light of about 1 Lux in the olfactometer. The experimental room was maintained at 27±1°C and a relative humidity of 61±9% RH. The temperature inside the flight chamber was 27±2.5°C and 64±9% RH. The air flowing out of the ports was maintained above 80% RH and an air temperature of 27.4±1°C.

Odor Stimuli Tested In The Olfactometer To insure a continuous and constant odor concentration for each compound within the odor plumes from start until the end of each experiment, we used air sample bags for ammonia, glass bottles for L-(+)-lactic acid, and 15 aliphatic carboxylic acids (Geier et al. 1999; Bosch et al. 2000).

Ammonia was supplied in a similar way as described in Smallegange et al. (2005). One day before the experiments, 250 µl of a 2.5% aqueous ammonia solution (25% in water; analytical grade, Merck) were injected into an 80 l Teflon air sample bag (SKC Gulf Coast Inc., Houston, TX, USA). Subsequently, the bag was filled with 60 l of warm, humidified, and charcoal filtered pressurized air at least 17 h prior to experiments to allow evaporation of the solution. This procedure resulted in an ammonia concentration of 136 ppm in the bag (Smallegange et al. 2005). During experiments, air pumps (Model 224-PCXR4, SKC Gulf Coast Inc., Houston, TX, USA) were used to lead air from the sample bag, through Teflon tubes (7 mm in diam; Rubber B.V., Hilversum, The Netherlands), and into the trapping devices at a flow of 230 ml/min. The flow of air was regulated by mechanical flow meters (Sho-Rate model GT1355; Brooks Instruments, Veenendaal, The Netherlands), and was mixed with the main air stream at a flow rate of approximately 23.5 l/min.

L-(+)-lactic acid (90% aqueous solution, analytical grade, Purac Bioquimica or 88–92% aqueous solution, Riedel-de Haën) (henceforth termed lactic acid) was mixed with the main air stream by tapping lactic acid vapor (10 ml) from a 250 ml glass bottle (Fisher Scientific B.V., ‘s Hertogenbosch, The Netherlands) with Teflon tubing. The flow rate was regulated to 15 ml/min by flow meters (Gilmont, Fisher Scientific B.V., ‘s Hertogenbosch, The Netherlands), which caused a lactic acid release comparable to that from a human hand (Smith et al. 1970; Geier et al. 1999). Since we applied lactic acid in a different way compared to previous experiments (Smallegange et al. 2005), we verified that it had no additional effect on the response of mosquitoes when added to ammonia.

Fifteen saturated aliphatic carboxylic acids (C2–C16) of the highest purity grade available were used in the experiments: >99% acetic acid (Sigma), 99% propanoic acid

(Sigma), 99% 2-methylpropanoic acid (Sigma), ≥99% 3-methylbutanoic acid (Sigma), >99% butanoic acid (Aldrich), >99% pentanoic acid (Sigma), ≥99% hexanoic acid (Sigma), 98% heptanoic acid (Sigma), ≥ 99% octanoic acid (Sigma), ≥ 97% nonanoic acid (Sigma), >99% decanoic acid (Sigma), ≥99% dodecanoic acid (Sigma), ≥ 98% tridecanoic acid (Sigma), >99% tetradecanoic acid (Sigma), ≥99% hexadecanoic acid (Sigma). Single, pure compounds [10 ml of a liquid compound (C2–C9) or 1 g of a solid compound (C10–C16)] were added to a 250 ml glass bottle (Fisher Scientific B.V., 's Hertogenbosch, The Netherlands). A charcoal-filtered, warm, humidified air stream was passed through the bottle and carried the vaporized compound at the desired flow rate through Teflon tubing and into the main stream through one of the trapping devices. Flow rates were regulated by Gilmont flow meters (Fisher Scientific B.V., 's Hertogenbosch, The Netherlands) at 0.5, 5, 50, and 100 ml/min. The calculated concentrations of the compounds in the air stream of the olfactometer are listed in Table S1 in the online supplement.

When tripartite blends were tested, the three compounds were mixed just before entering the trapping device. When more than one aliphatic carboxylic acid was part of the blend, a Perspex ring with 10 holes was attached upstream of each trapping device to be able to release each odor into the main air stream individually (Fig. 1). Tripartite blends were tested against ammonia alone. During experiments with multi-component blends, the multi-component odor blends were tested against the ammonia + lactic acid blend. Initially, we wanted to examine whether we could increase the attractiveness of ammonia—our best kairomone at that time. For this reason, we used ammonia alone as a control. In addition, we had observed that ammonia + lactic acid + a mixture of 12 carboxylic acids did not attract more mosquitoes than ammonia + lactic acid, but was more attractive than ammonia alone (Smallegange et al. 2005). Once we determined which individual carboxylic acids augmented the attractiveness of ammonia + lactic acid (Table 1), we continued our experiments with ammonia + lactic acid as the control, as we aimed for a better result than was found in Smallegange et al. (2005).

Olfactometer Tests Thirty female mosquitoes, 5–8 d-old, that had not received a blood meal, were randomly collected from their cage 14–18 h before the start of experiments. The mosquitoes were placed into a cylindrical release cage (8-cm diam, 10-cm high) with access to tap water from damp cotton wool placed on top of the cage. Experiments were performed during the last 4 h of the dark period, when *An. gambiae* is normally active (Haddow and Ssenkubuge 1973; Maxwell et al. 1998; Killeen et al. 2006).

In each trial, test compounds were released into the air stream and a group of mosquitoes was set free from a release cage placed at the downwind end of the flight chamber of the olfactometer, 1.60 m from the two ports. Mosquitoes were left in the flight chamber for 15 min. Female mosquitoes that had entered either of the trapping devices were counted at the end of the experiment, after anaesthetization with 100% CO_2. Mosquitoes remaining in the flight chamber were removed with a vacuum cleaner. After use, the trapping devices were washed with soapy water (CLY-MAX Heavy Duty Cleaner, Rogier Bosman Chemie B.V., Heijningen, The Netherlands), rinsed with tap water, and cleaned with cotton wool drenched in 70% ethanol (Merck).

The operator wore surgical gloves (Romed®, powderfree vinyl) to avoid contamination of the equipment with human volatiles. Each trial started with new mosquitoes and clean trapping devices. An experiment testing a particular blend was repeated at least 6 times on different days. The sequence of test odors was randomized on the same day and between days. Test stimuli were alternated between

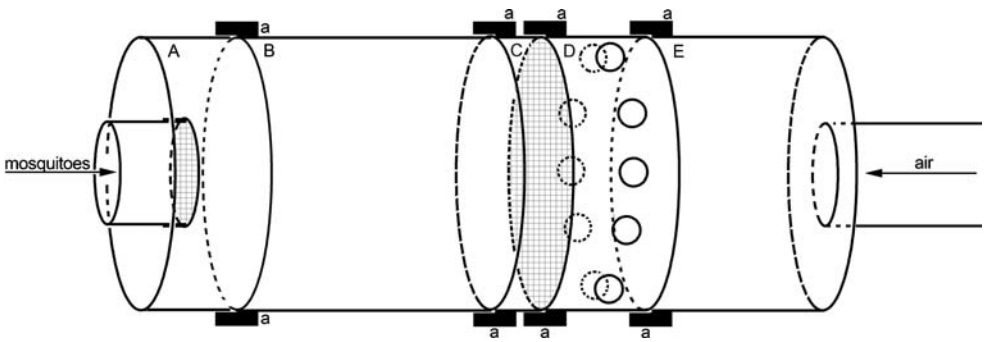

Fig. 1 An olfactometer trapping device is composed of three parts: *A*: part with baffle where mosquitoes enter the device; *B*: middle part device; *C*: distal end sealed with metal gauze to prevent mosquito crossing. A Perspex ring (*D*) with 10 holes for separate odor delivery. The end of the tube running from the glass bottle with an odor was inserted through one of the holes. Charcoal filtered, warm, humidified, pressurized air is led into the trapping device through *E* (a: schematic representation of the couplings between the various parts, made to fit smoothly on each other to prevent air loss)

Table 1 Effect of adding an individual carboxylic acid, at four flow rates (ml/min), to ammonia + lactic acid tested against ammonia alone in the dual-choice olfactometer. The result of the χ^2-test (*P*-value), trap entry response (%) and total number of mosquitoes released (n) are given for each two-choice test

Carboxylic acid	0.5ml/min	5ml/min	50ml/min	100ml/min
Acetic acid (C2)	*P*=0.25	*P*=0.24	*P*=0.85	*P*=0.56
	24.1%	22.5%	14.4%	15.3%
	n=199	*n*=262	*n*=201	*n*=177
Propanoic acid (C3)	*P*=0.13	*P*<0.001 A	*P*=0.59	*P*=0.001 A
	15.7%	21.2%	8.8%	20.8%
	n=178	*n*=156	*n*=160	*n*=154
2-Methylpropionic acid (2mC3)	*P*=1.00	*P*=0.38	*P*=0.34	*P*=0.22
	8.2%	18.9%	30.5%	39.9%
	n=170	*n*=175	*n*=174	*n*=198
Butanoic acid (C4)	*P*=0.03 A	*P*=0.69	*P*=0.53	*P*=0.47
	24.7%	11.6%	6.1%	11.0%
	n=150	*n*=225	*n*=164	*n*=155
3-Methylbutanoic acid (3mC4)	*P*=0.008 A	*P*=0.64	n.t.	n.t.
	16.6%	10.2%		
	n=169	*n*=176		
Pentanoic acid (C5)	*P*=0.13	*P*=0.32	*P*=0.86	*P*=0.01 A
	6.3%	20.6%	18.5%	39.4%
	n=174	*n*=175	*n*=178	*n*=175
Hexanoic acid (C6)	*P*=0.003 R	*P*=0.66	*P*=0.88	*P*=0.73
	19.7%	17.5%	26.3%	15.7%
	n=234	*n*=468	*n*=179	*n*=464
Heptanoic acid (C7)	*P*=0.03 A	*P*=0.005 R	*P*=0.007 R	*P*=0.85
	8.8%	11.0%	13.5%	15.4%
	n=226	*n*=164	*n*=170	*n*=175
Octanoic acid (C8)	*P*=0.30	*P*=0.47	*P*<0.001 A	*P*=0.23
	13.5%	16.0%	29.5%	29.2%
	n=170	*n*=187	*n*=193	*n*=195
Nonanoic acid (C9)	*P*=0.78	*P*=0.41	*P*=0.16	*P*=0.53
	7.6%	13.5%	4.5%	5.6%
	n=170	*n*=178	*n*=177	*n*=177
Decanoic acid (C10)	*P*=0.32	*P*=1.00	*P*=1.00	*P*=0.53
	4.1%	6.9%	3.7%	4.5%
	n=219	*n*=232	*n*=215	*n*=221
Dodecanoic acid (C12)	*P*=0.47	*P*=1.00	*P*=1.00	*P*=0.78
	9.4%	8.9%	4.4%	7.3%
	n=180	*n*=180	*n*=180	*n*=178
Tridecanoic acid (C13)	*P*=0.26	*P*=0.82	*P*=0.09	*P*=0.58
	23.4%	11.3%	25.4%	17.3%
	n=167	*n*=168	*n*=169	*n*=168
Tetradecanoic acid (C14)	*P*=0.02 A	*P*=0.04 A	*P*=0.007 A	*P*=0.01 A
	8.7%	11.2%	11.5%	12.7%
	n=173	*n*=170	*n*=174	*n*=173
Hexadecanoic acid (C16)	*P*=0.06	*P*=0.37	*P*=0.74	*P*=0.80
	5.7%	6.3%	5.2%	8.5%
	n=175	*n*=176	*n*=172	*n*=177

A: significantly more mosquitoes in the trapping device baited with the tripartite blend compared to the trapping device baited with ammonia (χ^2-test, $P<0.05$). R: significantly fewer mosquitoes in the trapping device baited with the tripartite blend compared to the trapping device baited with ammonia (χ^2-test, $P<0.05$). Calculated concentrations of the compounds in the odor plume are given in the online supplement (Table S1).

n.t. not tested

right and left ports in different replicates to rule out any positional effects. Experiments in which only clean air was fed into the olfactometer through both ports were done to test the symmetry of the trapping system.

Trapping Experiments in Screen Cage To assess the performance of some of the blends as a lure, we conducted laboratory experiments with volatile baited MM-X traps in a textile screen cage (233×250×330 cm; Howitec Netting BV, Bolsward, The Netherlands) inside a climate-controlled room (22.7±1.1°C and 52.4±7.4% RH). Two Mosquito Magnet-X (MM-X) traps (American Biophysics Corp., U.S.A.) (Kline 1999) were placed inside the cage at 2 m distance from each other.

Initially, a 9-compound blend (blend A) was tested. To compose this blend, 500 µl of a liquid pure compound or 500 mg of a solid compound (tetradecanoic acid) were put into individual low density polyethylene sachets (LDPE; 6×6 cm; Audion Elektro, The Netherlands; Torr et al. 1997) with a thickness of 0.1 mm (ammonia, lactic acid, heptanoic, octanoic, and tetradecanoic acid) or in a closed LDPE tube (32×14 mm, Kartell, 3.5 ml; Fisher Emergo, The Netherlands) within an LDPE sachet with a thickness of 0.2 mm (this delivery method was used for the 4 most volatile aliphatic carboxylic acids (i.e., propanoic, butanoic, 3-methylbutanoic, and pentanoic acids) to reduce their release to a higher extent than would have been possible with a sachet only). Only ammonia was diluted with distilled water (to 2.5%). The sachets were applied inside the central, black tube of the MM-X traps using odorless tape (3M™ Double Coated Tape 400). Air flow was created by a fan on top of this tube taking the headspace of the test blend downwards and outside the MM-X trap. Second, a tripartite blend was tested (blend B). Since we calculated that the release rates of the aliphatic carboxylic acids in blend A were at least two times higher than calculated for the multi-component blends tested in the optimization and subtraction olfactometer experiments, the 3 LDPE sachets (each with a thickness of 0.1 mm) containing the separate components of blend B were made as small as possible (2.5×2.5 cm) to reduce evaporation (Torr et al. 1997). One hundred µl of a liquid pure compound (ammonia, lactic acid) or 50 mg of a solid compound (tetradecanoic acid) were put into individual LDPE sachets. The amount of a compound within a sachet does not affect the evaporation rate of the compound through the LDPE material, whereas the surface of a LDPE sachet does (Torr et al. 1997). When blend B was applied, ammonia was not diluted (25.0%). Both blend A and B were tested against a blend of ammonia + lactic acid. Evaporation rates of the compounds were measured by weighing the LDPE sachets before and after experiments (see Table S1 in the online supplement).

Fifty female mosquitoes, 5–8 d-old, which had not received a blood meal, were randomly collected 14–18 h before the start of experiments. They were placed into a cylindrical release cage (diam 8 cm, height 17.5 cm) with access to tap water from damp cotton wool placed on top of the cage. The mosquitoes were set free from the release cage in the center of the screen cage. After 4 h, the MM-X traps were closed and transferred into a freezer to kill the mosquitoes. These experiments were performed during the last 4 h of the dark period. Each two-choice test was repeated either 4 or 6 times, alternating the position of each treatment every experimental day. Surgical gloves were worn to avoid contamination of equipment with human volatiles. Experiments with unbaited traps in the MM-X setup were done 6 times to test the symmetry of the trapping system.

Statistical Analysis For each two-choice assay, a Chi-square test was used to analyze whether the total number (i.e., sum of all replicates; a comparison of data collected on different days revealed no heterogeneity) of mosquitoes trapped in the treatment trapping device (of either olfactometer or MM-X trap) and the total number that was trapped in the control trapping device (of olfactometer or MM-X trap) differed from a 1:1 distribution. Effects were considered to be significant at $P<0.05$. The number of female mosquitoes caught in both trapping devices divided by the number of mosquitoes that flew out of the release cage is expressed as the trap entry response (TER).

Results

Initial Experiments The combined results of all dual-choice olfactometer experiments with clean air from both ports together show that equal numbers of mosquitoes were caught in both trapping devices (*Chi-square test, df*=1, $P=0.16$). This is proof that the olfactometer was symmetrical and that no positional bias interfered. Even when no volatile source was present, small numbers of mosquitoes flew into the trapping device. However, the TER was low (5.9%, $N=853$).

The combination of ammonia (136 ppm) + lactic acid (15 ml/min) caught mosquitoes in numbers similar to those caught by ammonia alone ($P=0.14$, $N=324$, TER=28.4%). Based on these results and the synergism between ammonia, lactic acid, and a blend of carboxylic acids that we reported previously (Smallegange et al. 2005), we decided to test single carboxylic acids by adding them to the blend of ammonia + lactic acid and examine the effect of this synthetic tripartite blend on the response of *An. gambiae* compared to the effect of ammonia alone.

Individual Aliphatic Carboxylic Acids First, 15 aliphatic carboxylic acids were tested in the dual-choice olfactometer at four different release rates, for their ability to increase the attractiveness of the blend of ammonia + lactic acid compared to ammonia alone. Seven of the carboxylic acids had no effect at any of the four concentrations (Table 1), namely acetic, 2-methylpropanoic, nonanoic, decanoic, dodecanoic, tridecanoic, and hexadecanoic acids (C2, 2mC3, C9, C10, C12, C13, and C16). Depending on the release rate applied, hexanoic acid had either no effect or significantly decreased the number of mosquitoes flying into trapping devices baited with the tripartite blend compared to those flying into the trapping devices baited with ammonia only (P=0.003 at the lowest concentration tested).

Adding propanoic, butanoic, 3-methylbutanoic, pentanoic, heptanoic, octanoic, or tetradecanoic acids (C3, C4, 3mC4, C5, C7, C8, and C14) to the ammonia + lactic acid blend resulted in an increase in the number of mosquitoes flying into the trapping device at one or more of the tested concentrations. The tripartite blend was more attractive than ammonia when butanoic, 3-methylbutanoic, pentanoic, heptanoic, or octanoic acids were part of the blend at either the lowest (flow rate 0.5 ml/min, C4, 3MC4 and C7, P<0.05), the second highest (50 ml/min, C8, P<0.001) or highest concentration (100 ml/min, C5, P=0.01). The second lowest and the highest concentrations of propanoic acid (5 and 100 ml/min) enhanced the attraction of ammonia + lactic acid ($P\leq$ 0.001). All four concentrations of tetradecanoic acid increased the attraction to ammonia + lactic acid compared with the attraction to ammonia alone (P<0.05). At the lowest concentration (0.5 ml/min), heptanoic acid increased the attractiveness of ammonia in combination with lactic acid (P=0.03). However, fewer mosquitoes were caught at the two intermediate heptanoic acid concentrations (5 and 50 ml/min) added to ammonia + lactic acid (P=0.005 and 0.007, respectively).

Optimization Of A Multi-Component Blend Based on previous odor blend experiments, a blend of ammonia + lactic acid and the 7 aliphatic carboxylic acids (C3, C4, 3mC4, C5, C7, C8, and C14) was prepared. This blend enhanced the attractiveness of ammonia when combined in a tripartite blend with lactic acid. Dual-choice olfactometer experiments were performed in which the concentrations of the 7 aliphatic carboxylic acids within this blend were varied in order to examine their effectiveness as mosquito attractants. In total, odor blends were tested in 6 different compositions in which the release rates varied between blends (see Table 2). Two of these experimental blends (blend 1 and blend 4) elicited significantly stronger attractions to the blend of 9 compounds (i.e., the 'complex blend') than to ammonia + lactic acid only (the 'control blend') (Table 2, P<0.001). The other blends were equally attractive as the control blend (Table 2, P>0.05).

Subtraction Experiments Blend 4, containing all 7 of the aliphatic carboxylic acids [each applied at the lowest dose (0.5 ml/min)], was selected for subtraction experiments. The blend minus one of the 7 acids was tested against the control blend (ammonia + lactic acid) to determine the contribution of each individual carboxylic acid on the attractiveness of the complex blend.

Removal of 3-methylbutanoic acid from the complex blend increased the blend attractiveness significantly (Table 3, P=0.01). When tetradecanoic acid was removed, the test blend attracted fewer mosquitoes into the trapping device than the control blend (P=0.02). Elimination of

Table 2 Optimization experiments with carboxylic acids in the dual-choice olfactometer. The left part of the table shows the flow rates for each individual aliphatic carboxylic acid within an odor blend consisting of nine components (ammonia + lactic acid + seven carboxylic acids). The total numbers of mosquitoes caught in the trapping device baited with the test blend (T) and in the trapping device baited with the control blend (C) are given. Also the result of the χ^2-test, total number of mosquitoes released (N) and trap entry response (TER) are given for each blend

Blend	Flow rate (ml/min)							T	C	χ^2-test	N	TER
	C3	3mC4	C4	C5	C7	C8	C14					
1	100	0.5	0.5	100	0.5	50	50	56	25	P<0.001 A	715	11.3%
2	50	0.5	100	0.5	0.5	100	100	3	2	P=0.65	169	3.0%
3	0.5	0.5	0.5	0.5	0.5	5	100	9	10	P=0.82	174	10.9%
4	0.5	0.5	0.5	0.5	0.5	0.5	0.5	30	9	P<0.001 A	548	7.1%
5	0.06	0.06	0.06	0.06	0.06	0.06	0.06	9	16	P=0.80	171	8.8%
6	5	0.5	0.5	5	0.5	5	5	13	5	P=0.06	146	12.3%

A: significantly more mosquitoes in the trapping device baited with the complex blend compared to the trapping device baited with ammonia + lactic acid (χ^2-test, P<0.05).

Table 3 Subtraction experiments performed in the dual-choice olfactometer. The total numbers of mosquitoes caught in the trapping device baited with the test blend (T) and in the trapping device baited with the control blend (C) are given. Also the result of the χ^2-test, total number of mosquitoes released (N) and trap entry response (TER) are given for each blend

Blend	T	C	χ^2-test	N	TER
Blend 4	15	15	P=1.000	338	8.9%
Blend 4 -C3	2	7	P=0.10	171	5.3%
Blend 4 -C4	7	10	P=0.47	164	10.4%
Blend 4 -3mC4	13	3	P=0.01 A	165	9.7%
Blend 4 -C5	1	6	P=0.06	171	4.1%
Blend 4 -C7	12	12	P=1.000	171	14.0%
Blend 4 -C8	9	6	P=0.44	160	9.4%
Blend 4 -C14	4	14	P=0.02 R	165	10.9%

A: significantly more mosquitoes in the trapping device baited with the complex blend compared to the trapping device baited with ammonia + lactic acid (χ^2-test, $P<0.05$).

R: significantly fewer mosquitoes in trapping device baited with complex blend compared to trapping device baited with ammonia + lactic acid (χ^2-test, $P<0.05$)

pentanoic acid may have reduced the attractiveness of the complex blend (P=0.06), but this effect was only marginally significant. Removal of the other four aliphatic carboxylic acids had no significant effect.

Effect of 3-Methylbutanoic Acid Based on the previous results, the effect of 3-methylbutanoic acid was examined by using a blend of ammonia + lactic acid + tetradecanoic acid (shown to have an important role within the complex blend, Table 3) and also propanoic and pentanoic acid (P-value in subtraction experiments $\leq$ 0.10, Table 3). In this series of experiments, blend 4 again was significantly more attractive than the control blend (ammonia+lactic acid) (Table 4, P=0.02). A blend of ammonia + lactic + propanoic + pentanoic + tetradecanoic acids was as attractive as the control (P=0.56). Adding 3-methylbutanoic acid to the latter blend at a flow rate of 0.5 ml/min did not alter the attractiveness, whereas reducing the flow rate to 0.05 ml/min increased attractiveness close to a marginally significant level (P=0.06).

Trapping Experiments Dual-choice experiments were conducted with MM-X traps baited with either a blend of ammonia + lactic acid + tetradecanoic acid or a blend of ammonia + lactic acid + the 7 carboxylic acids. These two blends were tested against the control blend containing ammonia + lactic acid. Traps baited with the tripartite blend and the 9-component blend trapped significantly more mosquitoes than traps baited with the control blend (Table 5, $P<0.001$). The two traps together caught 44.0% and 53.3% of the released mosquitoes (N=298 and 199, respectively). Two unbaited MM-X traps together caught 35.2% of the mosquitoes that left the release cage (N=585). These experiments without odor baits demonstrated that the system was symmetrical (P=0.40).

Discussion

Our results show that aliphatic carboxylic acids with defined chain lengths (C3–C8 and C14) enhance the attractiveness of ammonia to *An. gambiae* mosquitoes in a dose-dependent manner when applied in a tripartite blend with lactic acid. These compounds are likely to serve as kairomones in the host-seeking behavior of *An. gambiae*. As several of these compounds also affect the host-seeking behavior of other mosquito species—e.g., *Ae. aegypti* (Bosch et al. 2000) and *Culex quinquefasciatus* Say, another mosquito species that acts as a vector of human disease (Allan et al. 2006; Puri et al. 2006), our findings lend further credence to the kairomonal role of aliphatic carboxylic acids present on the human skin for anthropophilic mosquitoes.

Individual Aliphatic Carboxylic Acids The carboxylic acids that enhanced the attractiveness of the blend of ammonia and lactic acid to *An. gambiae* all occur in human skin

Table 4 Effect of adding four carboxylic acids in different combinations to ammonia + lactic acid tested against ammonia + lactic acid in the dual-choice olfactometer. The total numbers of mosquitoes caught in the trapping device baited with the test blend (T) and in the trapping device baited with the control blend (C) are given. Also the result of the χ^2-test, total number of mosquitoes released (N) and trap entry response (TER) are given for each blend

Blend	T	C	χ^2-test	N	TER
Blend 4	29	14	P=0.02 A	258	16.7%
ammonia + lactic acid + C3 + C5 + C14	15	12	P=0.56	346	7.8%
ammonia + lactic acid + C3 + $3mC4_{(0.5)}$ + C5 + C14	11	12	P=0.83	84	27.4%
ammonia + lactic acid + C3 + $3mC4_{(0.05)}$ + C5 + C14	22	11	P=0.06	261	12.6%

$3mC4_{(0.5)}$ and $3mC4_{(0.05)}$: 3-methylbutanoic acid applied at an air flow of 0.5 or 0.05 ml/min passing through the glass bottle containing this compound. All other aliphatic carboxylic acids were applied at a flow rate of 0.5 ml/min. A: significantly more mosquitoes in the trapping device baited with the complex blend compared to the trapping device baited with ammonia + lactic acid (χ^2-test, $P<0.05$).

Table 5 Blends tested in dual-choice screen cage experiments with MM-X traps. A MM-X trap was baited with one of the 2 test blends (T); the other MM-X trap was baited with ammonia and lactic acid (C). The total numbers of mosquitoes caught in the trap baited with the test blend (T) and in the trap baited with the control blend (C) are given. Also the result of the χ^2-test, total number of mosquitoes released (N) and trap entry response (TER) are given for each blend

Blend	T	C	χ^2-test	N	TER
A. ammonia + lactic acid + 7 carboxylic acids	85	21	$P<0.001$ A	199	53.3%
B. ammonia + lactic acid + tetradecanoic acid	87	44	$P<0.001$ A	298	44.0%

A: significantly more mosquitoes in the trapping device baited with the test blend compared to the trapping device baited with the control blend (χ^2-test, $P<0.05$). The release rate of each compound applied in LDPE sachets are given in the online supplement (Table S1).

emanations (Bernier et al. 2000; Healy and Copland 2000; Curran et al. 2005; Penn et al. 2007; Gallagher et al. 2008). Our results suggest that only few of these carboxylic acids, depending on their chain length and concentration, contribute to the attractiveness of *An. gambiae* to humans. Both attractive and repellent compounds might be involved in the differential host preference of this mosquito species (Qiu et al. 2004, 2006a). We have documented here that aliphatic carboxylic acids can act in both ways depending on concentration and molecular structure.

Knols et al. (1997) reported that 12 of the 15 acids tested also in this study comprised an attractive synthetic blend. However, the same blend was repellent to *An. gambiae* in a later study, but when combined with ammonia + lactic acid, produced an attractive blend in a synergistic fashion (Smallegange et al. 2005). Acetic acid levels in fresh sweat were higher than in incubated human sweat, the latter being more attractive to *An. gambiae* (Meijerink et al. 2000). Propanoic and hexanoic acids were detected in fresh sweat, but not incubated sweat. Levels of butanoic acid were similar in fresh and incubated sweat. 3-Methylbutanoic acid does occur in incubated human sweat, but in small amounts. We found that propanoic, butanoic, and 3-methylbutanoic acid affect the host-seeking response of *An. gambiae*.

At the lowest dose, heptanoic acid increased the attractiveness of ammonia + lactic acid, whereas fewer mosquitoes were attracted to the two intermediate doses (Table 1). This confirms that not only the molecular structure of the odor, but also its dosage determines whether an odor acts as an attractant or a repellent (Vale and Hall 1985). Heptanoic, nonanoic, and tridecanoic acids, the latter two inactive in this study, were not present in the 12 carboxylic acid-containing synthetic mixture used as attractant by Knols et al. (1997) and which had a synergistic effect in combination with ammonia and lactic acid (Smallegange et al. 2005).

A tripartite blend of ammonia + lactic + and hexanoic acid was found to have a synergistic effect in Y-tube olfactometer tests with *An. gambiae* (Smallegange et al. 2002). Olfactory neurons tuned to aliphatic carboxylic acids were found to innervate antennal sensilla trichodea in *An. gambiae* (Meijerink et al. 2000; Qiu et al. 2006b). Antennal grooved peg sensilla contain neurons sensitive to pentanoic and hexanoic acids. Hexanoic acid also elicited electrophysiological responses from olfactory neurons in trichoid sensilla (Qiu et al. 2006b). Carboxylic acids eliciting an electrophysiological response, but not showing a behavioral effect in this study, may be involved in other behaviors of this mosquito species.

C1–3, C5–8, and C13–18 carboxylic acids enhance the attractiveness of lactic acid to *Ae. aegypti* (Bosch et al. 2000). Several carboxylic acids (C2–18) attract high numbers of female *Cx. quinquefasciatus* in Y-tube olfactometer experiments or induce landing responses when tested individually (Allan et al. 2006; Puri et al. 2006). Bosch et al. (2000) found that hexanoic acid augmented the attractiveness of lactic acid to *Ae. aegypti*. A blend of ammonia, lactic acid, and hexanoic acid is now commercially available (BioGents, Germany; Kröckel et al. 2006) and shown to have potential as an attractant for *Ae. aegypti* (Kröckel et al. 2006; Williams et al. 2006a, b, c). However, in the present study with *An. gambiae,* none of the hexanoic acid doses enhanced attractiveness. In fact, repellency occurred at the lowest dose (Table 1). Attempts to reduce the hexanoic acid concentration from a glass bottle (0.05 ml/min and lower) did not have any effect on the attractiveness of the combination of ammonia, lactic acid, and hexanoic acid compared to ammonia alone. Also, flow rates of 25 ml/min and 230 ml/min had no effect (data not shown). When 8 doses of hexanoic acid were applied to a sand-blasted glass slide (see Smallegange et al. 2005), no attraction was found (in combination with ammonia and lactic acid against ammonia and solvents (diethyl ether; Merck, ≥99.8%) to dilute hexanoic acid (Sigma, ≥99%), and ethanol (Merck, ≥99.8%) to dilute L(+)-lactic acid (sodium salt, Sigma, 98%) (data not shown). Therefore, we conclude that hexanoic acid is not important to *An. gambiae* in attracting host-seeking females from a distance.

Several carboxylic acids showed neither an attractive nor a repellent effect toward *An. gambiae* females at any of the concentrations tested. We do not know the actual concentra-

tion of the acids encountered by mosquitoes in our bioassays or when engaged in host-seeking at varying distances from the host under typical host-seeking conditions. It is possible that the acids were tested at concentrations (see Table S1 in the online supplement) outside of the range that naturally occur in the volatile blends released by humans. Further study of the absolute concentration of human-produced volatiles in the odor plume is required to clarify the role of single carboxylic acids as kairomones for blood feeding insects.

Optimization Experiments Both the optimization and the trapping experiments with the odor blends consisting of nine compounds showed that the blend containing ammonia, lactic acid, and the 7 carboxylic acids enhanced the effect of ammonia in the first experiment, and is attractive to *An. gambiae*. However, the concentration and/or the ratio of carboxylic acids, are crucial factors as can be concluded from the optimization experiments (Table 2). Apparently, the flow rates of the carboxylic acids in blend 5 were too low to induce an effect. When comparing blend 1, 2, and 4, it is likely that the flow rates of butanoic, octanoic, and/or tetradecanoic acid in blend 2 were too high. It is surprising that blend 3 showed no effect. This suggests that the flow rate of tetradecanoic acid was too high, although this seems to contradict the results obtained with this carboxylic acid in the first experiment. It may be that the highest concentration at which tetradecanoic acid is attractive depends on the presence of other carboxylic acids.

Subtraction Experiments And The Effect Of 3-Methylbutanoic Acid Blend 4 was no longer more attractive than the control blend when we initiated the series of subtraction experiments (Table 3), unlike the results presented in Table 2. However, the subtraction experiments are valid, since the effect of eliminating an individual aliphatic carboxylic acid on the attractiveness of the complex blend is clear. In addition, blend 4 was attractive in a series of olfactometer experiments conducted after the subtraction studies (Table 4). Moreover, MM-X traps baited with this blend attracted 4 times more mosquitoes than the control blend in a larger set-up (Table 5). Therefore, we conclude that blend 4 is attractive to *An. gambiae* and that the blend components jointly affect host-seeking behavior.

Individual removal of propanoic, butanoic, pentanoic, heptanoic, or octanoic acid had no effect on the attractiveness of the complex blend suggesting that these 5 carboxylic acids do not play a significant role in the attractiveness of the complex blend and might be exchanged by one another.

Elimination of tetradecanoic acid made the complex blend less attractive than the control blend, whereas the complex blend without 3-methylbutanoic acid was significantly more attractive than the control blend. This suggests that 3-methylbutanoic acid has a repellent or inhibitory effect. On the other hand, a low dose of 3-methylbutanoic acid (0.05 ml/min) also appeared to have a positive effect (although not quite significant) compared with the blend of 5 compounds (Table 4). The effect of 3-methylbutanoic acid seems to depend on the dose of this compound and the other carboxylic acids in the blend (see also Table 1). However, strictly adhering to the significance level of 5%, none of the blends with either 3 or 4 aliphatic carboxylic acids mentioned in Table 4 had the same effect as blend 4 (P=0.02). In other words, these four aliphatic carboxylic acids cannot replace the 7 aliphatic carboxylic acids in blend 4 to enhance the attractiveness of ammonia + lactic acid. Apparently (taking all results together), interactions between aliphatic carboxylic acids in odor blends affect the "active" composition of the blend, which we cannot fully elucidate based on our results. Combining the subtraction results with the result of the first experiment where tetradecanoic acid increased the attractiveness of ammonia when lactic acid was present at all flow rates, suggests that it is an important component in behaviorally significant odor blends for *An. gambiae*. This aliphatic carboxylic acid was also found to be more abundant (almost twice as much) in the odor profiles of two people that were more attractive to *An. gambiae* than two people with lower amounts of tetradecanoic acid. In addition, tetradecanoic acid was a major constituent in the odor profiles of these four people as determined by sampling skin residues on small glass beads (Qiu et al. 2006a). The residues were desorbed by placing the beads into a thermal desorption unit combined with a cryo-focussing system and then analyzed by GC-MS (A.M. Galimard, personal communication).

Trapping Experiments The trapping experiments showed that a complex blend of 9 compounds is more attractive than the control blend (ammonia + lactic acid), which is in agreement with the results of the olfactometer assays. A catch rate of about 42.7% of the mosquitoes released by the MM-X trap baited with the complex blend compared to 17.6% per trap in experiments with two unbaited traps is a promising finding. Semi-field and field experiments should be conducted to reveal the potential of this blend as a bait for wild mosquitoes in Africa. The MM-X trap baited with the tripartite blend also caught more mosquitoes than the MM-X trap baited with the bipartite blend, suggesting that despite its low volatility, tetradecanoic acid increases the attractiveness of ammonia and lactic acid in two different assays.

Comparisons of the release rates that were calculated based on physicochemical parameters and the gravimetrically determined evaporation rates (Table S1 in the online supplement) of compounds applied in the olfactometer and in the MM-X traps, respectively, seem to show differences

in the concentrations applied in the two bioassays. However, it is hard to estimate the exact concentrations encountered by mosquitoes, considering the differences between the two bioassays (e.g., sizes of the experimental arenas in which experiments have been conducted and the release methods used for the test compounds).

The tripartite blend caught about 29.2% of the total number of mosquitoes released into the experimental room. When the attractiveness of the 9-component blend is compared statistically with the tripartite blend, a significant difference is not found [P=0.73; GLM (binomial, link in logit); calculated for the ratio of (treatment)/(treatment + control); mean 0.60±0.39 (9-component blend) and 0.66± 0.11 (tripartite blend)]. Thus, the trapping experiments show, in addition to olfactometer data, that tetradecanoic acid in combination with ammonia and lactic acid may be used as a reliable basic blend to which other candidate kairomones for *An. gambiae* can be added. Although semi-field and field experiments have to be conducted to see how well this tripartite blend performs under African conditions, it seems likely that the blend efficacy can be improved by adjusting the concentrations of each compound and by addition of compounds other than aliphatic carboxylic acids (R.C. Smallegange, unpublished data).

In summation, our results demonstrate that several aliphatic carboxylic acids elicit a significant odor-mediated behavioral preference in the malaria mosquito *An. gambiae*. They also enhance the attractive effectiveness of the ammonia and lactic acid mixture. Tetradecanoic acid emerges as a crucial compound and is attractive over a broad concentration range. In contrast, 3-methylbutanoic acid reduced the trap entry response when present in a blend of ammonia, lactic acid and seven carboxylic acids. The inhibitory effect was much less when the dose was reduced and applied in a blend with 6 compounds. The behavioral effect of 3-methylbutanoic acid needs to be further examined. A remarkable species-specific difference was revealed when hexanoic acid was found to act as an inhibitor or to be ineffective, whereas in the anthropophilic *Ae. aegypti* it is an important kairomone. Semi-field and field studies are needed to confirm that the qualitative and quantitative composition of attractive blends for *An. gambiae* reported here can be successfully employed in trapping strategies under field conditions.

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Insects use a combination of visual and chemical cues to locate their host plants (Bernays and Chapman 1994), and plant volatiles play an important role (Bruce et al. 2005). There is evidence that *H. hampei* uses both visual (Morallo-Rejesus and Baldos 1980; Mathieu et al. 2001) and olfactory stimuli (Giordanengo et al. 1993; Mendoza-Mora 1991; Mathieu et al., 2001). However, to date, no studies have established which volatiles are responsible for attraction of *H. hampei*, possibly because the small size of the antennae makes electrophysiological recordings a challenge. The ripeness/physiological maturity of berries of coffee determine the level of attraction and colonization by the beetle (Baker 1999). Studies conducted so far have demonstrated that female insects prefer (ripe) or black (overripe) berries over green ones (Giordanengo et al. 1993; Mendoza-Mora 1991; Baker 1999; Mendesil et al. 2005). The volatile composition of coffee berries at different stages of ripeness has been reported (Mathieu et al. 1996, 1998; Warthen et al. 1997; Ortiz et al. 2004), but no electrophysiological studies of responses to coffee volatiles have been conducted previously with *H. hampei*, and the semiochemicals used in host location have not yet been defined. Identification of the volatile compounds used for host recognition may contribute to novel and sustainable pest management strategies, for example by using baited traps. To address this, the objective of the present study was to identify volatiles released by coffee berries that elicit electrophysiological responses from *H. hampei* antennae, and to investigate behavioral responses to these semiochemicals in an olfactometer.

Methods and Materials

Insects The culture of coffee berry borer used in the laboratory experiments was established with field collected insects from Ethiopia. They were maintained under controlled conditions (21°C, 75% RH, 12:12 h L:D) and reared in the laboratory on artificial media as described by Brun et al. (1993).

Chemicals Authentic samples of methylcyclohexane, nonane, ethylbenzene, (*R*)-limonene, (*S*)-limonene, and octen-3-ol were purchased from Sigma Aldrich (Gillingham, UK; all≥96% purity). 3-Ethyl-4-methylpentanol was provided by Prof J. Millar, Department of Entomology, University of California.

Collection of Volatiles Coffea arabica (Rubiaceae) berries were collected in Ethiopia (local coffee type, from garden coffee near Jimma town). Berries at green, ripe, and dry stages (500 g) were enclosed separately in a glass vessel (20 cm length, 6 cm diam). Air entering the vessel was drawn through a charcoal filter. Air was pumped out at 400 ml min^{-1} through a Porapak Q adsorbent tube (Alltech associates, Lancashire, UK) in a 5 mm diam glass tube (Alltech associates, Carnforth, Lancashire, UK; 50 mg Porapak Q in 5 mm diam glass tube). All connections were made with PTFE tubing (Alltech associates, Lancashire, UK) with brass ferules and fittings (North London Valve, London, UK) and sealed with PTFE tape (Gibbs & Dandy, Luton, UK). Berries were entrained for 3 d, and the Porapak Q filter was eluted with 0.5 ml of redistilled diethyl ether, providing a solution that contained the isolated volatile compounds. Samples were stored in the freezer (−22°C) until used for the experiments.

Gas Chromatography Analysis Volatiles were analyzed on a Hewlett-Packard 6890 GC equipped with a cold on-column injector and a flame ionization detector (FID) fitted with two columns of different polarity: a non-polar HP-1 bonded-phase fused silica capillary column (50 m× 0.32 mm i.d., film thickness 0.52 µm) and a polar DB-wax column (50 m × 0.32 mm i.d., film thickness 0.82 µm). The oven was maintained at 30°C for 1 min and then programmed at 5°C min^{-1} to 150°C followed by 10°C min^{-1} to 230°C. The carrier gas was hydrogen. Chiral GC analyses were conducted by using an HP5890 GC (Agilent Technologies, UK) equipped with a cool on-column injector and a FID, fitted with a β-cyclodextrin chiral capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; 140 kPa head pressure). The GC oven was maintained at 50°C for 1 min, and then raised by 2°C min^{-1} to 150°C where it was held for 30 min. The carrier gas was hydrogen.

Coupled Gas Chromatography-Electroantennography (GC-EAG) Electroantennogram (EAG) recordings were made by using Ag-AgCl glass microelectrodes filled with Ringer solution (7.55 g l^{-1} sodium chloride, 0.64 g l^{-1} potassium chloride, 0.22 g l^{-1} calcium chloride, 1.73 g l^{-1} magnesium chloride, 0.86 g l^{-1} sodium bicarbonate, 0.61 g l^{-1} sodium orthophosphate). The head of an adult female *H. hampei* was separated from the body with a microscalpel and laid upside-down on double-sided sticky tape, then secured with a water-based erasing fluid (Tippex, BIRO BIC Ltd.) placed around the base of the head and allowed to dry. The antennae were brought forward and secured in a similar way. The indifferent electrode, from which the tip had been removed, was placed within the head capsule. The recording electrode was inserted into the tip of one antenna. The coupled GC-EAG system, in which the effluent from the GC column is simultaneously directed to the antennal preparation and the GC detector, has been described previously (Wadhams 1982). The stimulus was delivered into a purified and humidified airstream (1 l/min) flowing continuously over the preparation. Separation of the

volatiles was achieved with a Hewlett-Packard 6890 GC equipped with a cold on-column injector and a flame ionization detector (FID). The column was HP-1 (50 m× 0.32-mm i.d.). The oven temperature was maintained at 30°C for 2 min, and then programmed at 5°C min^{-1} to 100°C and then at 10°C min^{-1} to 250°C. The carrier gas was hydrogen. EAG signals were passed through a high-impedance amplifier (UN-06; Syntech, the Netherlands), and simultaneous recordings of the EAG and FID responses were obtained with specialized software (EAD version 2.3; Syntech, the Netherlands). Six coupled runs were completed. Only FID peaks that corresponded to an EAG peak in three or more replicates were considered to be electrophysiologically active.

Coupled Gas Chromatography-Mass Spectrometry (GC-MS) Identification of electrophysio-logically active FID peaks was achieved by GC-MS. One µl of the air entrainment sample was injected onto a capillary GC column (50 m×0.32 mm i.d. HP1) directly coupled to a mass spectrometer (Thermo-Finnigan, MAT95, Bremen, Germany). Ionization was achieved by electron impact at 70 eV, 250°C. The oven temperature was maintained at 30°C for 5 min, and then programmed at 5°C min^{-1} to 250°C. Tentative identifications of electrophysiologically active FID peaks were made by comparison of spectra with those of authentic samples in a database (NIST 2005). Tentative identification by GC-MS were confirmed by co-injection of the air entrainment sample with authentic standards on both HP-1 and DB-WAX columns, with peak enhancement indicating co-elution. Quantification of identified compounds was achieved by injecting authentic standards at three known concentrations onto the HP 1 GC column and recording the peak area. Each standard was injected three times, and a calibration curve was plotted for each compound, and used to determine quantities of each compounds in the air entrainment sample.

Olfactometer Bioassay Behavioral assays were done by using a Perspex four-arm olfactometer (Pettersson 1970). It was illuminated from above by diffuse uniform lighting (23 µmol $m^{-2}s^{-1}$) and maintained at 23°C. The bottom of the apparatus was lined with filter paper (Whatman No 1), and air was drawn through the four arms towards the center at 240 ml min^{-1} (flow rate through each individual arm was 60 ml min^{-1}). Single female *H. hampei* (1 – 3 wk-old, assumed mated as obtained from a mixed culture) were introduced into the central chamber, and the time spent and number of entries into each arm was recorded using specialist software (OLFA, Udine, Italy) over a 16-min period. The apparatus was rotated a quarter of a turn every 2 min to eliminate any directional bias. If the insect remained motionless continuously for 2 min, it was considered inactive, and the replicate was repeated by using a new insect. After each experiment, all glassware was washed thoroughly with Teepol detergents, rinsed with acetone and then with distilled water and baked in an oven for at least 2 h. Perpex components were washed with Teepol solution, rinsed with 80% ethanol solution, and then with distilled water and left to air dry.

Initially, responses to headspace samples collected from green, ripe, and dry stages of *C. arabica* berries were tested. Ten µl of headspace sample were applied to a filter paper strip, and the solvent was allowed to evaporate for 30 s. The filter paper was placed at the end of the treated side arm. The three control arms were similarly treated with redistilled solvent (diethyl ether) (10 µl) on filter paper. The sample that elicited the strongest behavioral response was selected for GC-EAG analysis, and electrophysiologically active synthetic compounds then were used as treatments in subsequent bioassays. Responses to individual compounds were tested by using 10 µl of 100 ng $µl^{-1}$ solutions formulated in redistilled hexane in the treated arm and 10 µl of redistilled hexane in the control arm. Compounds that elicited a positive behavioral response were formulated as a synthetic blend (in redistilled hexane) that comprised all the active compounds in the same concentration and ratio as in the entrainment sample they were identified from, and the response to 10 µl aliquots of this blend was tested. For each series of replicates, the mean time spent in and number of entries into treated and control arms were compared using a paired *t*-test (GenStat) after checking that data were normally distributed. A choice test also was conducted in which there were two treated arms and two control arms (Webster et al. 2008). Thus the response to the synthetic blend and the natural headspace sample it was based on were directly compared.

Results

Behavioral Responses of H. hampei to Headspace Samples In the olfactometer bioassay, female *H. hampei* spent more time in the region of the olfactometer where ripe and dry stage fruit volatiles were used as an odor source compared to control regions ($P<0.01$). However, the insects showed no significant response to odor from green stage *C. arabica* berries (Fig. 1). The entrainment sample of dry berries that gave the strongest response in the bioassay ($P=0.005$) was selected for use in coupled GC-EAG recordings.

Identification of Electrophysiologically Active Compounds in Air Entrainment Sample Coupled GC-EAG revealed 6 electrophysiologically active compounds in the headspace sample. By using coupled GC-MS and GC peak enhance-

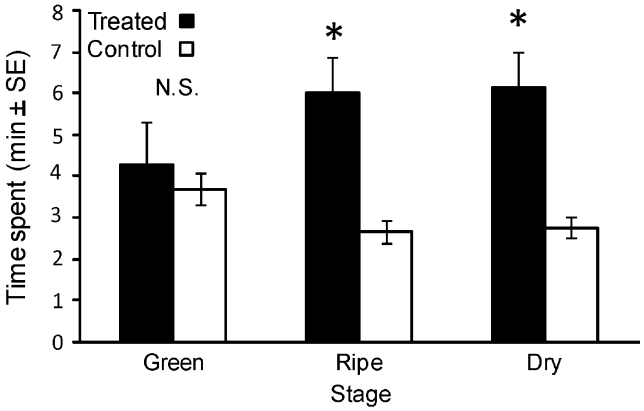

Fig. 1 Behavioral responses of female coffee berry borer, *Hypothenemus hampei* to green, ripe and dry stage of *Coffea arabica* berries in four-arm olfactometer (N=8) (*=P<0.01)

ment on two GC columns of different polarity, these 6 compounds were identified as methylcyclohexane, ethylbenzene, nonane, 1-octen-3-ol, limonene, and 3-ethyl-4-methylpentanol. The quantities and ratio of these compounds are listed in Table 1. Chiral GC analyses indicated that the main enantiomer of limonene was (*R*)-limonene, although a trace of (*S*)-limonene also was present. Quantities of 1-octen-3-ol were too small in the natural sample to resolve the enantiomer. For 3-ethyl-4-methylpentanol, a racemic synthetic sample gave two peaks eluting at 23.03 and 23.36 min, whereas the natural sample of *C. arabica* berry volatiles only gave one peak eluting at 23.06 min. The earlier eluting peak was enhanced in a co-injection of natural and synthetic samples. The (*R*)-enantiomer is known to elute before the (*S*)- enantiomer on a ß-cyclodextrin column (Greenberg et al. 2007), and thus the compound present was (*R*)-3-ethyl-4-methylpentanol.

Amounts of Electrophysiologically Active Compounds in Samples from Different Growth Stages GC analysis of samples collected from green stage berries indicated that few volatiles were emitted at this phenological stage. Out of the electrophysiologically active compounds, only methylcyclohexane was detected. More volatiles were emitted at the ripe stage (Table 2), with methylcyclohexane and (*R*)-3-ethyl-4-methylpentanol being released in maximum amounts. At the dry stage, there was a decrease in release of methylcyclohexane and (*R*)-3-ethyl-4-methylpentanol, and ethylbenzene, nonane, octen-3-ol, and (*R*)-limonene formed a greater proportion of the volatile blend (Table 2).

Behavioral Responses of H. hampei to Synthetic Compound Of the six electrophysiologically active compounds, four elicited attraction when presented individually in the olfactometer. Female *H. hampei* showed significant responses to 3-ethyl-4-methylpentanol (P=0.005), nonane (P=0.021), methylcyclohexane (P = 0.029), and ethylbenzene (P=0.042) (Fig. 2). There was no significant response to (*R*)-limonene and octen-3-ol. When 3-ethyl-4-methylpentanol, nonane, methylcyclohexane, and ethylbenzene were combined in a 4-component synthetic blend, in the same concentration and ratio as the natural headspace sample, they elicited a highly significant response (P< 0.001) (Fig. 2). A choice test between this 4-component blend and the natural headspace sample showed that there was no significant difference in time spent (P= 0.229). The mean time (± S.E.) in the area where insects were exposed to the synthetic blend was 3.20 (± 0.77) min, while it was 3.63 (± 0.20) min in the area where they were exposed to the natural sample of volatiles.

Discussion

Female coffee berry borer were attracted to ripe and dry stage *C. arabica* berry volatiles in the olfactometer bioassay (P<0.01) (Fig. 1), whereas volatiles collected from green immature berries were not significantly attractive. There were clear differences in volatile blends released at the different phenological stages (Table 2), especially at the green stage where all but one of the electrophysiologically active compounds were not detected, thus explaining the lack of a behavioral response to green stage headspace samples. This agrees with previous reports of the preference of this insect for ripe and over ripe *C. arabica* berries

Table 1 Electrophysiologically active compounds identified in *Coffea arabica* berry volatiles

Compound no.	Compound name	Retention Index (polar)	Concentration[a] (ng µl^{-1})	Ratio[a]
1	methylcyclohexane	752	33.9	9.1
2	ethylbenzene	852	10.3	2.8
3	nonane	902	3.7	1.0
4	1-octen-3-ol	951	5.0	1.3
5	(*R*)-3-ethyl-4-methyl pentanol	1010	40.3	10.8
6	(*R*)-limonene	1023	53.0	14.2

[a] Concentration and ratio as in the sample of dry stage berry volatiles that elicited the greatest response in olfactometer bioassays

Table 2 Amounts of electrophysiologically active compounds in headspace samples collected from green, ripe and dry stage *Coffea arabica* berries[a]

Compound no.	Compound name	Mean Concentration (ngµl^{-1}) (± S.E.)		
		Green stage	Ripe stage	Dry stage
1	methylcyclohexane	3.20 (± 2.88)	254 (± 234)	9.69 (± 9.31)
2	ethylbenzene	n.d.	0.71 (± 0.45)	6.59 (± 2.77)
3	nonane	n.d.	0.20 (± 0.20)	2.98 (± 1.50)
4	1-octen-3-ol	n.d.	0.22 (± 0.22)	2.98 (± 1.60)
5	(*R*)-3-ethyl-4-methyl pentanol	n.d.	2595 (± 1814)	24.3 (± 13.2)
6	(*R*)-limonene	n.d.	3.22 (± 2.08)	21.4 (± 14.7)

n.d. not detected

[a] N=4

(Mendoza-Mora 1991; Giordanengo et al. 1993; Mendesil et al. 2005). The small size of the insect meant that electrophysiological studies with the antennae reported here were somewhat challenging. Nevertheless, six electrophysiologically active compounds were identified in a headspace sample of dry (over ripe) *C. arabica* berries. Of these, methylcyclohexane, ethylbenzene, nonane, and 3-ethyl-4-methylpentanol were significantly attractive when presented individually in the olfactometer bioassay. A blend of these four key compounds, in the same concentration and ratio as in the headspace sample they were identified from, elicited highly significant attraction of *H. hampei* ($P < 0.001$), suggesting that the identified volatile compounds are used in host location by *H. hampei*. (*R*)-Limonene and octen-3-ol were not significantly attractive when presented alone, and were not crucial for the response observed to the headspace sample, as the 4-component synthetic blend not containing them was as attractive as the natural headspace sample in a choice test.

Two of the four compounds identified in this study as attractants for *H. hampei* have been reported previously: Ortiz et al. (2004) found nonane and 3-ethyl-4-methylpentanol in headspace analyses of *C. arabica* berries. However, methylcyclohexane and ethylbenzene have not been reported previously. Methylcyclohexane has been found in the essential oil of *Laportea aestuans* (Olufunke et al. 2008). Ethylbenzene is in a range of plants including *Limonium bicolor* (Wei and Wang 2006), poplar bark (Zhao et al. 2002), olives (Scarpati et al. 1993), Malabar nightshade (Kameoka et al. 1991), and *Cucurbita* flowers (Granero et al. 2004). Nonane is in an even wider range of plants other than *C. arabica*. 3-Ethyl-4-methylpentanol is a more unusual compound, and could perhaps be used to distinguish *C. arabica* from non-host material, as it is less widely occurring. However, even this compound is not unique to *C. Arabica*, as it has been reported as a component of French bean (*Phaseolus vulgaris*) volatiles (Barra et al. 2007) and as a trace compound in wine aroma (Genovés et al. 2005; Zhang et al. 2007). It also has a different function in a different ecological context as a sex pheromone of the ant *Polyergus breviceps* (Greenberg et al. 2007). Other compounds emitted in significant quantities, but not found to be electrophysio-

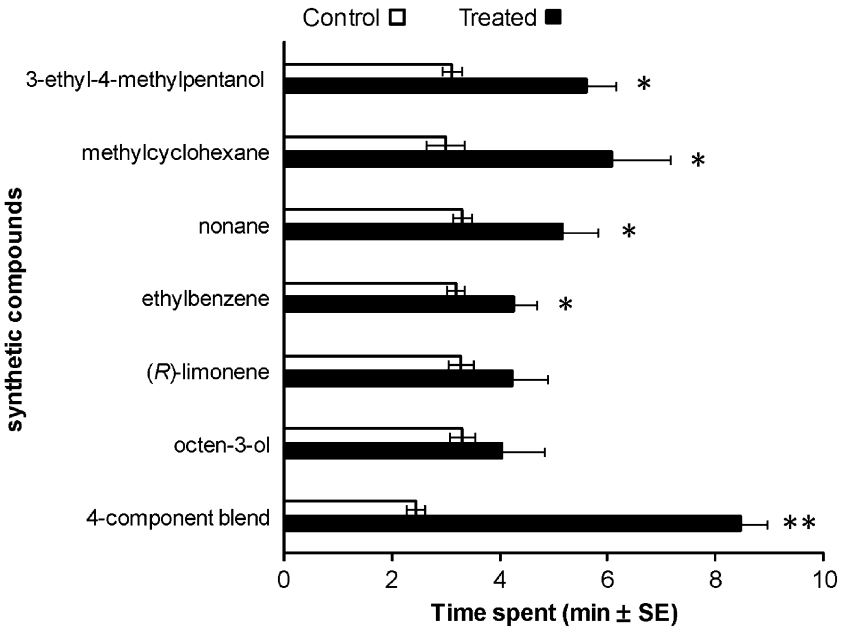

Fig. 2 Behavioral responses of female coffee berry borer, *Hypothenemus hampei* to electrophysiologically active synthetic compounds (100 ng on filter paper) and a four-component blend of synthetic compounds in four-arm olfactometer (N=8) (*=P<0.05; **=P<0.001)

logically active, were hexanal, 1-hexanol, 2-heptanone, 2-heptanone, 2-heptanol, isobutyl butyrate, linalool, and methyl salicylate, and they have been previously identified in coffee berries (e.g., Mathieu et al. 1998).

As the attractants identified here are not specific to *C. arabica*, it is unlikely that a single volatile compound is used for host recognition by *H. hampei*. We suspect that *H. hampei* uses a blend of compounds for host recognition because the response in the olfactometer to a 4-component blend of volatiles that comprised methylcyclohexane, ethylbenzene, nonane, and 3-ethyl-4-methylpentanol in the natural ratio elicited considerably stronger attraction than any of these compounds presented individually (Fig. 2). Use of a blend can enable host recognition by host specific ratios of ubiquitous rather than host-specific volatile compounds (Visser 1986; Bruce et al. 2005). Further studies are required to elucidate fully the role of ratios in host location by *H. hampei* and to define parameters of the minimum component blend required for attraction in the field. Furthermore, to study whether there are any herbivore induced volatiles emitted from *C. arabica* fruits, as in the case with other insect species (Dicke 1994; Karban and Baldwin 1997; Jönsson and Anderson 1999), would add a valuable dimension to the work. Most importantly, field trials should be carried out to assess the potential for use of the semiochemicals identified here in integrated pest management of the coffee berry borer. Baited traps could be useful for monitoring pest abundance (including for quarantine) or even in mass trapping as female insects were attracted to the semiochemicals in the olfactometer.

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expression of other genes, and interfering with apoptosis (Eaton and Gallagher 1994; McLean and Dutton 1995; Neal 1995; Guengerich et al. 1998). Economic losses due to aflatoxin contamination are estimated at millions of dollars in the U.S. each year. To minimize the harmful effects of aflatoxins, strict regulations have been established in the US. While crops and finished food products that contain aflatoxins above 20 ppb are not allowed for human consumption, the limitation is set at 5 ppb for milk (Cleveland et al. 2003; Williams et al. 2004). Two other fungi, *Aspergillus ochraceus* and *Penicillium verrucosum,* primarily produce ochratoxins. Ochratoxin A (OTA) interferes with immune system function (Neal 1995) and also may be carcinogenic. Because of these properties, commodities contaminated with high concentrations of mycotoxins (e.g., 20 ppb for aflatoxins) are blocked from sale both in the U.S. and overseas (Park and Troxell 2002; Bayman and Baker 2006). Without appropriate monitoring measures, mycotoxin-contaminated food has had serious impacts on human health in many areas, especially in developing countries in Asia and Africa (Williams et al. 2004).

The level of tolerance to mycotoxins in insects varies from several ppb to several ppm and is both species- and sex-specific (Gudauskas et al. 1967; Chinnici et al. 1976; Llewellyn and Chinnici 1978; Chinnici and Llewellyn 1979; Zeng et al. 2006). *Drosophila melanogaster* larvae, which feed naturally on fermenting fruits, exhibit significantly altered development on diets containing low AFB1 concentrations (i.e., less than 1 ppm); larvae feeding on higher concentrations will die (Kirk et al. 1971; Chinnici et al. 1976; Llewellyn and Chinnici 1978; Chinnici and Llewellyn 1979). The corn earworm, *Helicoverpa zea* (CEW), another species that encounters mycotoxin-releasing fungi in damaged plants (Widstrom et al. 1976; Widstrom 1979; Archer and Bynum 1994), also is tolerant of aflatoxins. Low concentrations of AFB1 in the diet of 1st instars (less than 100 ng/g) can affect development, causing delayed pupation and reduced pupal weight; higher concentrations of AFB1 (200 ng/g) are lethal (Zeng et al. 2006).

The toxicity of aflatoxins to insects depends on their metabolism as mediated by cytochrome P450 monooxygenases (P450s, Phase I detoxification enzymes) into bioactive products. One of the most toxic of these is the AFB1-*exo*-epoxide, which is derived from AFB1. The bioactivated metabolite of AFB1, exo-8,9-epoxide (AFBO), can bind directly to proteins and DNA and cause cell death (Eaton and Gallagher 1994; McLean and Dutton 1995; Guengerich et al. 1998; Suriawinata and Xu 2004). Glutathione-S-transferases (GSTs, Phase II detoxification enzymes) conjugate glutathione to the lipophilic products of P450-mediated metabolites that are more readily excreted (Hayes et al. 2005). In rodent livers, susceptibility to AFB1 is inversely associated with GST activity toward AFBO (Lotlikar et al. 1984; Monroe and Eaton 1988; Buetler et al. 1992; Johnson et al. 1997). Another biotransformation of AFB1 is its direct reduction by NADPH-dependent aflatoxin B1 aldehyde reductases (AFAR) to form aflatoxicol (AFL). However, this product is converted rapidly back to AFB1 by dehydrogenases (Eaton and Gallagher 1994). Alternative biotransformation pathways that generate detoxified metabolites, such as aflatoxicol, aflatoxin M1, and aflatoxin B2a, and that do not generate bioactivated epoxidized metabolites also exist in NOW and contribute to the high aflatoxin tolerance of this species (Lee and Campbell 2000). Characterization of similar biotransformation pathways in CEW indicate that at least one P450 in this species, specifically CYP321A1, is capable of converting AFB1 into polar metabolites, with the demethylated product aflatoxin P1 (AFP1) as the principal metabolite (Niu et al. 2008).

Given the existence of alternative biotransformation pathways in both NOW and CEW, we initiated a series of studies to compare toxicity and tolerance of mycotoxins in these two species. Because NOW is associated regularly with fungus-contaminated hostplant tissue, we hypothesized that its level of tolerance to both aflatoxins and ochratoxins should exceed that of CEW, which only occasionally encounters fungal contamination inasmuch as it feeds on fruits before they fall to the ground.

Methods and Materials

Chemicals Piperonyl butoxide (PBO) was purchased from Tokyo Kassie Kogyo (Tokyo, Japan). Aflatoxins B1, G1, and ochratoxin A were purchased from Sigma Chemicals (St. Louis, MO, USA). Analytical grade dimethyl sulfoxide (DMSO) was obtained from Fisher Scientific (Pittsburgh, PA, USA). All chemicals were stored at −20°C and dissolved in DMSO to make stock solutions.

Insects Laboratory colonies of *H. zea* (originating from specimens collected in Champaign County, IL, USA), and *A. transitella* (originating on almond trees in Fresno, CA, USA that have been maintained in culture for the past 25 years by the Commodity Protection and Quality Unit, USDA/ARS, Parlier, CA, USA) were kept in an insectary at UIUC at 28±4°C with 16-h/8-h L/D cycle. *H. zea* larvae were reared individually from egg hatch in 5 oz (142 g) plastic cups containing approximately 5 g control or supplemented diet. *A. transitella* larvae were mass-reared until pupation in 500 ml plastic containers containing 200 g of wheat bran diet (Tebbets et al. 1978).

Bioassays AFB1 and OTA s*tock* solutions were prepared in DMSO and stored at −20°C for up to 2 months. Diets containing AFB1 (1, 5, 10, 20, 50, and 100 μg/g) were made from AFB1 stock solutions (1, 100, or 500 μl of 1 mg/ml in 0.5% DMSO) and incorporated into 100 g of diet to make 1 or 5 μg/g of AFB1 diet; 50, 100, 250 and 500 μl of 20 mg/ml of AFB1 stock solution were added to 100 g of the diet to make 10, 20, 50 and 100 μg/g of AFB1 diets in 0.5% DMSO. For low concentrations of AFB1 diets (i.e., 20, 40, 60, 80, 100, 120, or 140 ng/g), AFB1 stock solution in 0.2% DMSO (20, 40, 60, 80, 100,120, 140 μl of 100 μg/ml) was added to 100 g of diet. OTA stock solutions (50 or 250 μl of 2 mg/ml in 0.25% DMSO) were incorporated into 100 g of the OTA–containing diet to make 1 or 5 μg/g of the OTA diet; in addition, OTA (50, 100, or 250 μl of 20 mg/ml in 0.25% DMSO) was added to 100 g of the diet to make 10, 20, or 50 μg/g of the OTA diet. Pilot experiments determined that concentrations of DMSO up to 0.5% of the diet do not affect development or survivorship in either NOW or CEW.

To assess the toxicity of AFB1 to NOW, six concentrations of AFB1 (1, 5, 10, 20, 50, and 100 μg/g) diets were prepared. NOW neonates (20 newly hatched 1st instars) were placed individually into 142-g plastic cups that contained 0.5 g unamended diet or supplemented diet prepared with a final concentration of 0.5% DMSO. Two higher concentrations of AFB1-containing diets (50 and 100 μg/g) were fed to 20 ultimate instars (5th) with 0.5% DMSO as control. Larvae were monitored until pupation, and final pupal weights were measured within 48 h after the final larval molt. Each set of bioassays was replicated three times.

Similarly, OTA toxicity to NOW larvae was tested with a variety of OTA-containing diets (1, 5, 10, 20, or 50 μg/g OTA at a final concentration in 0.25% DMSO); 1st instars (20) in each group were treated. On the 12th day, the proportion of dead larvae was recorded, and each larva was weighed on the 14th day. The pupation rate was measured within 48 h of pupation. To test the toxicity of OTA to CEW, 1st instars were exposed to diets containing 1 or 5 μg/g OTA in DMSO (0.25%) and control diet. The survivorship of larvae at 12 days post-hatch on diets and frequency of 5th instars at 12 days post-hatch were recorded.

To compare the effects of AFB1 on the development of NOW and CEW, 1st instar CEW larvae were fed artificial diets containing 0.2% DMSO and 10, 20, 40, 60, 80, 100, 120, or 140 ng/g AFB1, while 1st instar *A. transitella* were fed with artificial diets containing 0.5% DMSO, 1, 5, or 10 μg/g AFB1. For each species, the numbers of larvae reaching the 2nd instar were recorded within 48 h.

Midgut Protein Preparations To obtain sufficient larval midgut tissue, guts were dissected from ultimate instars (at least 100) that had molted within the previous 12 h. Midgut dissections were performed in 0.1 M phosphate buffer and on ice. The midguts were frozen in liquid nitrogen and stored at −80°C until use. For each midgut preparation of ultimate instars, frozen midguts (approx. 40) were ground in a mortar in the presence of liquid nitrogen. The resulting material was transferred into a Eppendorf tube (1.5 ml), suspended in phosphate buffer (1.5 ml; 0.1 M; pH 7.4), EDTA (1 mM), PMSF (0.5 mM), DTT (0.1 mM), and 20% glycerol; the mixture was shaken gently for 5 s. These midgut homogenates were centrifuged (10,000 rpm; 10 min) in an Eppendorf centrifuge at 4°C. The cleared supernatant was transferred into a new 1.5 ml Eppendorf tube for immediate use, or frozen in liquid nitrogen and stored at −80°C for up to 6 months. Protein concentrations for the cleared lysates were determined by using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

In Vitro Metabolism Assays For all assays, the frozen cleared lysates were taken from the −80°C freezer and thawed on ice (approx. 30 min) before the metabolism assays were initiated. AFB1 and OTA metabolism assays were carried out as described previously in Lee and Campbell (2000) with only slight modification. Stock solutions of AFB1 or OTA at 10 μg/g were prepared in DMSO. Each 250-μl reaction contained AFB1 (1 μl) or OTA stock (10 μg/μl), reduced glutathione (5 mM), protein (1 mg/g), sodium phosphate buffer (0.1 mmol/l; pH 7.4), and EDTA (1 mM). Reactions were initiated with the addition of 50 μl of 3 mM NADPH. After a 60-min incubation period (at 30°C with shaking), 1 ml of ice-cold methanol containing 10 μM AFG1 (internal standard) was added to terminate reactions. The reaction mixtures were centrifuged (10,000 g for 10 min) in an Eppendorf centrifuge, and each supernatant was analyzed by high-pressure liquid chromatography (HPLC). All reactions were run in triplicate and repeated at least twice. To trap the AFB1-*exo*-epoxide metabolite, mouse liver cytosol (10 μl, 50 mg/ml) was added into reactions containing NOW crude proteins. Reactions were stopped after incubation (30°C for 1 h) and analyzed (10 μl) by HPLC.

AFB1 and its metabolites were separated by HPLC using a reverse-phase Supercosil LC-18 column (250×4.6 mm), a flow rate of 1 ml/min, and were detected at 362 nm with photodiode array UV detector (Waters Novapak). A ternary solvent system consisting of water/methanol/acetonitrile (60:20:20) was used to separate AFG1 (eluted at 10.6 min) and AFB1 (eluted at 14.6 min). Separation of OTA was achieved with an acetic acid/methanol/acetonitrile (1/49/49) solvent system, at a flow rate of 1 ml, and monitoring at 333 nm. OTA eluted at 10.4 min.

Statistics All data were analyzed by using a one-way ANOVA with a post-hoc Tukey test at a significance level of $P<0.05$.

Results

Aflatoxin B1 And Ochratoxin A Toxicity to NOW Larvae *Amyelois transitella* larvae tolerated high dietary levels of AFB1, with concentrations of 1, 5, 10, and 20 μg/g AFB1 having no apparent toxic effect on 1st instars (Table 1). In diets containing 10 and 20 μg/g AFB1, pupation rates were 77% and 78%, respectively. These did not differ significantly from pupation rates on control DMSO diet, and there was no significant difference in pupal weights between diets containing 10 and 20 μg/g AFB1 and control diets. On the diet containing 50 μg/g AFB1, larval development was delayed and only 55% of the larvae successfully pupated, a rate significantly lower than the rate with the control diet ($P<0.05$). At the extremely high AFB1 concentration of 100 μg/g, development of 1st instar *A. transitella* was severely inhibited; however, 30% of the treated larvae survived for at least 3 weeks. Exposure of later stage larvae to diets containing varying concentrations of AFB1 showed that their tolerance to AFB1 increased with developmental stage; ultimate instars tolerated 100 μg/g AFB1 with no significant decreases in pupation rate compared to control larvae fed diets containing 0.5% DMSO (Table 2).

Similarly, *A. transitella* 1st instars also were tolerant of OTA, with concentrations less than 10 μg/g having no detectable toxic effect (Table 3). At higher OTA concentrations (20 μg/g), there were no significant differences in mortality rates or larval weight after 14 days. Compared with control larvae fed diets with 0.25% DMSO, *A. transitella* fed with the highest concentration of OTA (50 μg/g) experienced slight effects on development, as evidenced by lower larval weights; larval weights after 14 days were significantly lower than those fed control diet ($P=0.047$). The highest concentration of OTA (50 μg/g) did not lower the pupation rate (Table 3).

Comparison of OTA and AFB1 Toxicity of to CEW and NOW The toxicity of AFB1 in *A. transitella* and *H. zea* was measured to compare the effect of AFB1 on 1st instars. The LC_{50} for AFB1 *A. transitella* (defined as the concentration preventing 50% of newly hatched larvae from molting to 2nd instar within 48 h) is 100 times greater for than for *H. zea* (Fig. 1). After 48 h, 60 ng/g AFB1 allowed 50% of 1st instar *H. zea* to develop into 2nd instars, while 10 μg/g AFB1 allowed 80% of 1st instar *A. transitella* to develop to 2nd instars—a greater than 100-fold difference in tolerance.

Table 1 Pupation rate and pupal weight of *Amyelois transitella* larvae after exposure to different concentrations of aflatoxin B1 (in 0.5% DMSO) at 1st instar. Values for pupation rate are means ± SE from 20 1st instar caterpillars/treatment with 3 experimental replicates for each. Values for pupal weight are representative of one of the 3 series of replicates and are means with SE

Concentration (μg/g)	Pupation rate (%)	Pupal weight (mg)
0.5% DMSO	85±6	43±2
1	90±5	44±2
5	85±6	45±2
10	77±9	45±2
20	78±2	44±3
50	55±10[a]	40±5
100	0	0

[a] Indicates that the mean value of this group is significantly different from that of the control group (0.5% DMSO) ($P<0.05$)

Table 2 Toxicity of aflatoxin B1 to ultimate (5th) instar *Amyelois transitella.* Values for pupation rate are means ± SE from 20 ultimate instars/treatment with 3 experimental replicates for each. Values for larval and pupal weight are representative of 1 of the 3 series of replicates and are means with SE. The data were evaluated with one-way analysis of variance (ANOVA), and no differences were found in the means of pupation rate or pupal weight among the groups

Concentration (μg/g)	Pupation rate (%)	Pupal weight (mg)
plain diet	97±6	41±4
0.5% DMSO	98±3	38±3
50	92±10	38±4
100	93±3	39±4

Table 3 Toxicity of ochratoxin A to 1st instar larvae of *Amyelois transitella.* Values for survival rate are means ± SE from 3 replicate bioassays. Values for larval and pupal weight are representative of one of the three series of replicates and are means with SE.

Concentration	Survival rate on day 12 (%)	Larval weight on day 14 (mg)	Pupation rate (%)
Plain diet	90±10	38±2	90±7
0.25%	85±5	36±2	75±4
1	80±10	32±9	80±7
5	85±5	33±3	83±5
10	85±10	35±5	75±4
20	90±5	33±2	78±2
50	80±5	26±6[a]	75±4

[a] Indicates that the mean value of this group is significantly different from that of the control group (0.5% DMSO) ($P<0.05$)

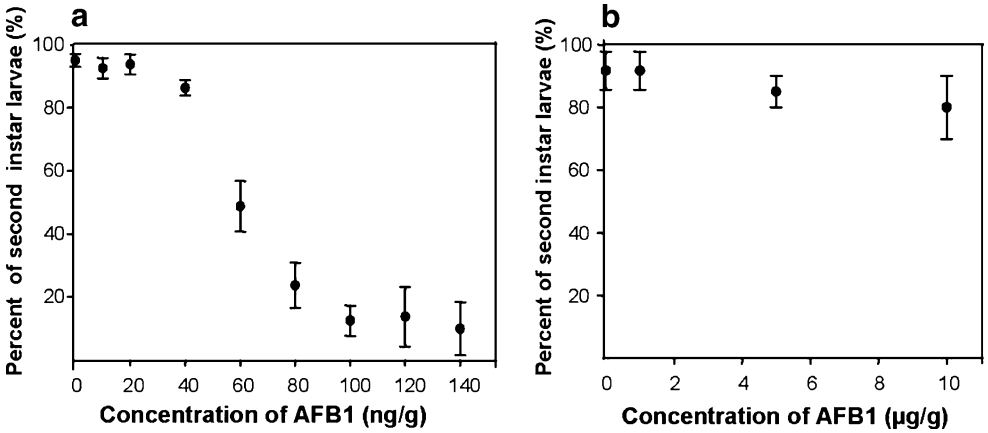

Fig. 1 Developmental effects of AFB1 on 1st instar *Helicoverpa zea* and *Amyelois transitella*. **a** Percentage of *H. zea* larvae molting to 2nd instar after 48 h on diets containing increasing concentrations of AFB1 and a final concentration of 0.2% DMSO. **b** Percentage of *A. transitella* larvae molting to 2nd instar after 48 h on diets containing increasing concentrations of AFB1 and a final concentration of 0.5% DMSO

Comparisons of the toxicity of OTA to *A. transitella* and *H. zea* also revealed considerably greater resistance to OTA in *A. transitella*. Development of 1st instar *A. transitella* was not affected by concentrations as high as 1 and 5 µg/g OTA. These concentrations significantly inhibited the development of 1st instar *H. zea* compared with control larvae, with 10% or no 5th instars appearing after 10 days on 1 or 5 µg/g OTA-supplemented diets, and 40% 5th instars appearing on the 0.25% DMSO control diet (P= 0.032 or 0.015, respectively) (Fig. 2).

NOW Metabolism of AFB1 and OTA To investigate the ability of *A. transitella* to metabolize AFB1, *in vitro* metabolism experiments were conducted with cleared midgut lysates quantified based on total protein. In reactions initiated with NADPH (as outlined in Methods and Materials), two polar metabolites of AFB1 (Met1 with R_t=7.8 min and Met2 with R_t=8.0 min) were readily detected by HPLC analysis (data not shown). Compared with *H. zea* midgut lysates, *A. transitella* midgut lysates have higher turnover rates (32±4 pmol/min/mg total protein vs. 0 pmol/min/mg total protein). In contrast, CEW midguts show no activity toward AFB1 unless induced by prior exposure to coumarin and xanthotoxin (Niu et al. 2008).

In an attempt to trap reactive AFBO intermediates, mouse liver cytosol was added to some *in vitro* reactions, but no additional metabolite peaks were detected (data not shown). This finding is consistent with the suggestion that AFB1 is detoxified, and not bioactivated, as the result of hydroxylation by midgut proteins. Comparable reactions with OTA showed no OTA metabolism by *A. transitella* or *H. zea* lysates either at the level of substrate disappearance or metabolite production. The methods used in this analysis may have been insufficiently sensitive to detect metabolism; alternatively, both of these species may rely on detoxification in tissues other than midgut, or non-enzymatic means, to counter the toxic effects of this compound.

Discussion

Zeng et al. (2006) and Niu et al. (2008) studied the toxicity and metabolism of AFB1 by *H. zea*, a lepidopteran with tolerance to a broad range of phytochemicals but only limited tolerance to aflatoxins even though it occasionally encounters aflatoxin-releasing fungi. In contrast, *A. transitella*, a herbivore that specifically locates and feeds on unharvested fruits, is tolerant of extremely high concentrations of mycotoxins in its diet. Our studies indicate that *A. transitella*, although a hostplant generalist, may well be highly specialized for mycotoxin detoxification. Indeed, recent studies indicate that NOW grows better on fungus-contaminated hostplant tissue and in fact may consume fungal tissue as well (Palumbo et al. 2008; personal observations).

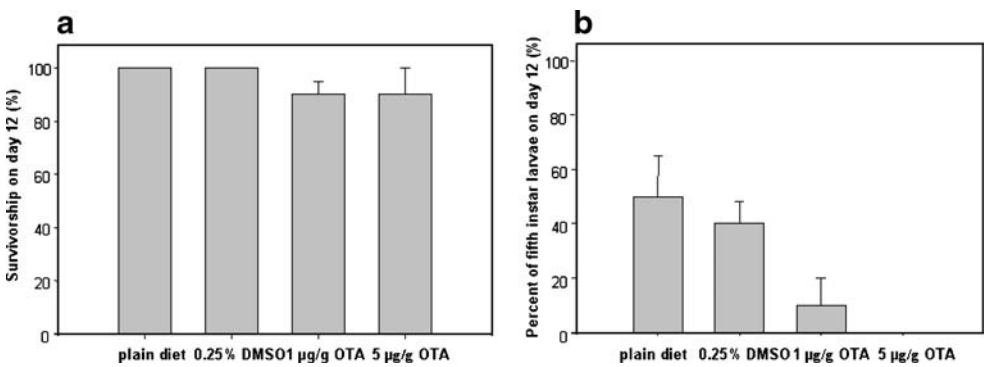

Fig. 2 Toxicity of OTA to 1st instar larvae of *Helicoverpa zea*. **a** Survivorship of larvae 12 days post-hatch on diets containing increasing concentrations of OTA and a final concentration of 0.25% DMSO. **b** Frequency of 5th instar larvae 12 days post-hatch on diets containing 0.25% DMSO, 1 µg/g OTA, or 5 µg/g OTA

In this study, we demonstrated the extremely high tolerance of *A. transitella* to concentrations of AFB1 and OTA and showed that this tolerance exceeds that of other mycotoxin-associated species such as *H. zea*. The concentrations analyzed did not kill *A. transitella* when administered at early developmental stages and caused only marginal sublethal effects. Comparisons of the toxicological effects in *A. transitella* and *H. zea* demonstrated that *A. transitella* larvae are 100 times more tolerant of AFB1 than *H. zea* and also are significantly more resistant to OTA. The ecological consequences of this tolerance are apparent in the high survival rates of *A. transitella* larvae in the mycotoxin-contaminated environments that it exploits as a scavenger. The interaction between NOW and *Aspergillus* species might be mutualistic in that the fungus may contribute to maintaining a micro-environment that optimizes caterpillar growth and development, and the caterpillar may promote the dispersal and establishment of the fungus by causing damage that allows fungal spores to gain access to fruits (Weis 1982). *Aspergillus* species can be cultured from adults, and the levels are high in adults emerging from *Aspergillus*-infested substrate (JS, unpublished data).

Aflatoxin tolerance might be explained by an excess detoxification capacity that generates nontoxic metabolites. Both Lee and Campbell (2000) and this study demonstrated that bioactivated metabolites are not produced in the reactions of NOW proteins. Because AFBO production increases toxicity 100-fold compared to its parent compound (AFB1) (Eaton and Gallagher 1994; McLean and Dutton 1995; Guengerich et al. 1998; Suriawinata and Xu 2004), absence of AFBO production by NOW might be a key feature of AFB1 tolerance. In contrast with AFB1, there are no reports of insect detoxification of OTA. Although we failed to detect metabolites of OTA produced by midgut proteins of either *H. zea* or *A. transitella*, bioactivation is involved in its genotoxicity to other organisms (Manderville 2005; Schaut et al. 2008), and the absence of major pathways that produce bioactivated metabolites also may reflect adaptation of these insects to the presence of this mycotoxin in their diet.

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mitochondria, which resulted in production of two new isoprenoids and enhanced attraction of carnivorous predatory mites (*Phytoseiulus persimilis*) (Kappers et al., 2005). We have set out to modify the glucosinolate profile in *A. thaliana* in order to study tritrophic interactions between the plant, an insect pest (*Brevicoryne brassicae*) and its natural enemy, the braconid endoparasitoid (*Diaeretiella rapae*). Cruciferous plants (Brassicaceae) characteristically accumulate glucosinolates, a group of amino acid-derived secondary metabolites consisting of a β-thioglucose moiety, a sulfonated oxime, and a variable side chain. Together with endogenous myrosinase (β-thioglucoside glucohydrolase, 3.2.1.147) glucosinolates serve a central role in defense against herbivores and pathogens (Wink, 1988; Jander et al., 2001; Kliebenstein et al., 2005a, b). Tissue damage brings together myrosinase and glucosinolates, which are otherwise spatially separated (Kelly et al., 1998) yielding a variety of toxic hydrolysis products (Fig. 1), such as isothiocyanates, epithionitriles, thiocyanates, and nitriles (Bones and Rossiter, 1996, 2006; Halkier and Gershenzon, 2006).

The myrosinase-glucosinolate system is an effective defense against many generalist insect herbivores. However, specialist crucifer-feeding insects have evolved counter adaptive biochemical mechanisms to overcome this defense. This is achieved, in the larvae of the cabbage white butterfly, *Pieris rapae* (Wittstock et al., 2004), and the diamondback moth, *Plutella xylostella* (Ratzka et al., 2002), through different types of detoxification mechanisms. Other insect herbivores, including larvae of the turnip sawfly, *Athalia rosae* (Muller et al., 2001), the harlequin bug, *Murcantia histrionica* (Aliabadi et al., 2002), as well as the cabbage aphid, *Brevicoryne brassicae*, and the turnip aphid, *Lipaphis pseudobrassicae* (= *L. erysimi*) (Bridges et al., 2002; Kazana et al., 2007) appear to tolerate glucosinolates, and actually sequester these compounds from their host plants. Sequestration of glucosinolates provides these insects with a defense against potential natural enemies. In the two species of aphid, a myrosinase, distinct from plant myrosinase, has evolved, resulting in a defense that mimics that of their host plants, with the production of isothiocyanates following tissue damage (Jones et al., 2001, 2002; Bridges et al., 2002; Husebye et al., 2005). Isothiocyanates provide both a direct, toxic, defense against potential natural enemies (Francis et al., 2001), and an indirect defense by synergizing the response of aphids to the alarm pheromone, *E*-β-farnesene (Dawson et al., 1987). Despite this defense, both *B. brassicae* and *L. pseudobrassicae* are subject to attack by a range of natural enemies, such as insect parasitoids, which also apparently are adapted to toxic glucosinolate hydrolysis products (Bridges et al., 2002).

Exploitation of the cruciferous plants' myrosinase-glucosinolate defense by *B. brassicae* is not restricted to glucosinolate accumulation. Volatile cues, in the form of isothiocyanates are also utilized by the aphid in host-plant location (Nottingham et al., 1991). Similarly, several studies have shown that the braconid wasp, *D. rapae*, a solitary endoparasitoid of aphids that feed on crucifers, is able to exploit glucosinolate hydrolysis products as olfactory cues in host foraging behavior. These olfactometry studies recorded positive behavioral responses of female parasitoids to several species of *Brassica* experimentally infested with aphids, including cabbage (*Brassica oleracea*) (Reed et al., 1995), turnip (*Brassica rapa* var *rapifera*) (Blande et al., 2007), and collard (*Brassica oleracea*) (Read et al., 1970), as well as the model crucifer *A. thaliana* (Girling et al., 2006). Additional olfactometry experiments, using glucosinolate hydrolysis products in place of aphid infested plants identified isothiocyanates as important signaling chemicals (Read et al., 1970; Reed et al., 1995; Vaughn et al., 1996; Bradburne and Mithen, 2000; Blande et al., 2007). Air entrainments further confirmed that higher levels of isothiocyanates were produced by aphid infested turnip plants compared with uninfested plants, and the ability of *D. rapae* females to discriminate between these two odor sources (Blande et al., 2007). Interestingly, parasitoids were not able to discriminate between plants infested with the specialist *L. pseudobrassicae* or the generalist *Myzus persicae*, despite the fact that once the aphid host is located, *D. rapae* attacks the crucifer-specialist at a greater rate than the generalist (Blande et al., 2004).

Tritrophic interactions are inherently complex, and the impact of specific compounds is often difficult to discern (Burow et al., 2006; Barker et al., 2007). Plants that differ in their glucosinolate or glucosinolate hydrolysis profile may also differ in other ways, such as the presence of other deterrents, stimulants, or synergists, leaf morphology, or nutritional content (Nielsen et al., 2001). These concerns may be addressed partly through the use of pure compounds and artificial diets, although this approach is less successful in defenses where toxic compounds are formed

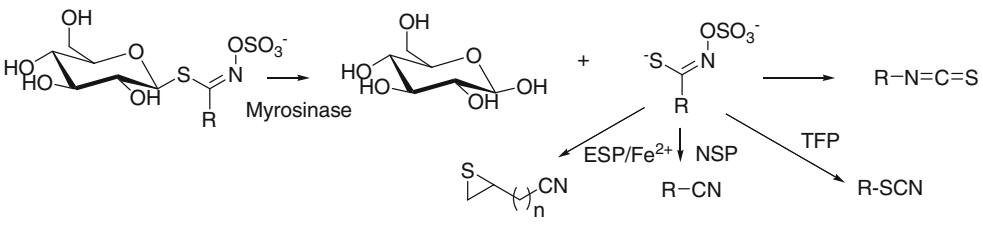

Fig. 1 Glucosinolate hydrolysis pathway. ESP: epithiospecifier protein; TFP: thiocyanate forming protein; NSP: nitrile-specifier protein

from inactive precursors, such as in the myrosinase-glucosinolate system (Wittstock et al., 2003).

The genetic characterization of model plants such as *A. thaliana* offers a potentially powerful tool with which to assess the importance of specific compounds in both bitrophic and tritrophic interactions. This approach has been applied to investigate the indirect roles of signaling pathways (Mewis et al., 2006) and a novel calmodulin-binding protein (Levy et al., 2005) on insect herbivory through their impacts on glucosinolate levels, while other studies have looked at the effects of elevated glucosinolate levels (Nielsen et al., 2001). A number of studies have focused on glucosinolate hydrolysis and the impact of epithiospecifier protein (ESP) (Jander et al., 2001; Lambrix et al., 2001; Burow et al., 2006) and the associated epithiospecifier modifier1 (ESM1) (Zhang et al., 2006). Other studies have focused on the functional significance of the myrosinase part of this defense (Barth and Jander, 2006).

There have been few studies looking at the impact of modifying the glucosinolate profile by transgenic methodology on plant-herbivore interaction, although recently the effect of overexpressing the dioxygenase AOP2 in Col-0 on a generalist pest was determined (Hansen et al., 2008). Variation in methionine-derived aliphatic glucosinolate side-chain modification in *A. thaliana* is controlled by the *GS-AOP* locus, which has three alleles that produce methylsulfinylalkyl (*GS*-null), alkenyl (*GS-ALK*), or hydroxyalkyl (*GS-OHP*) side-chains (Kliebenstein et al., 2001b, 2005a). The functional alleles code for 2-oxoglutarate-dependent dioxygenases that determine the nature of the aliphatic glucosinolate profile by converting the methylsulfinylalkyl functionality to alkenylglucosinolates (AtAOP2) or to hydroxyalkylglucosinolates (AtAOP3). The Columbia (Col) ecotype, however, does not possess a functional *GS-AOP*, and consequently accumulates 4-methylsulfinylbutylglucosinolate. Thus, by overexpressing a 2-oxoglutarate dependant dioxygenase in Col-5, it is possible to switch the plant from a methylsulfinylalkylglucosinolate producer to one with alkenylglucosinolates (Fig. 2). Here, we present responses of the crucifer specialist, *B. brassicae* and its natural enemy, *D. rapae*, to wild-type Col-5 plants and to Col-5 plants transformed with a functional *AtAOP2* ortholog from *Brassica nigra* (*BniGSL-ALK*). We also compare the response of the parasitoid to the *A. thaliana* ecotype Ru-0, which produces 2-propenylisothiocyanate.

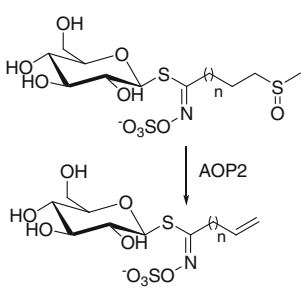

Fig. 2 Biosynthesis of the alkenyl glucosinolates from the methylthioalkyl precursor. AOP2: 2-oxoglutarate dependent dioxygenase

Methods and Materials

Plant Material and Growth Conditions Seeds of the *Arabidopsis thaliana* (L.). Heynh. ecotypes Col-5 and Ru-0 were obtained from the Nottingham Arabidopsis Stock Centre (NASC). Seeds were sown on wet soil (Levington F2 compost:vermiculite; 4:1) and cold treated for 2 d at 4°C to get homogenous germination. Seedlings were pricked out 2 wk after cold treatment and transferred to individual 40×40×50 mm plastic plant cells. Plants were grown under short day conditions in a controlled environment chamber (12 h light: 12 h dark regime, 100–150 µmol $m^{-2}s^{-1}$ during light period; 22°C during light and 20°C during dark).

Cloning of an Ortholog of the Arabidopsis thaliana 2-oxoglutarate Dependent Dioxygenase 2 (AtAOP2) from Brassica nigra An ortholog of the *A. thaliana* gene coding for the 2-oxoglutarate dependent dioxygenase 2 (*AtAOP2; At4g03060*) was cloned from *B. nigra* (L.) Koch genomic DNA by PCR using *Pwo* DNA polymerase (Roche Applied Science) according to the supplier's instructions. We named this ortholog *BniGSL-ALK*.

The oligonucleotide primers (MWG Biotech, Ebersberg, Germany) used for amplification of *BniGSL-ALK* were designed on published sequences from *Brassica oleracea*: the genomic DNA sequence *BoGSL-ALK* (AY044425; Li and Quiros, 2003) and the corresponding region of clone B21H13 (AC122543). The sequence of the forward primer was 5'-*atcc***catg**ggtgcagacacttctcaacttc-3' (in bold: start codon; underlined: *Nco*I site; in italics: additional triplet of an *Eco*RV restriction site) and that of the reverse primer was 5'-gcgcacgtg**tta**tgctccagagacg-3' (in bold: stop codon; underlined: *Pml*I restriction site).

For cloning purposes, the environment of the original translation initiation site, contained in the forward primer, was changed to create an *Nco*I restriction site, and an additional 5'ATC-triplet of an *Eco*RV restriction site also was included. A *Pml*I restriction site was included in the reverse primer. The PCR amplified product was cloned into *Eco*RV- restricted pBluescript SK+and partially sequenced.

Ectopic Expression of BniGSL-ALK in Arabidopsis thaliana An *Nco*I/*Pml*I-restricted fragment containing *BniGSL-ALK* was excised from the pBluescript construct, inserted into the binary vector pCAMBIA3301, where it is under the

control of a CaMV35S promoter, and the integration sites of the insert were verified by sequencing. The *BniGSL-ALK* expression construct was transferred into *Agrobacterium tumefaciens* (strain GV3101) by electroporation (Sambrook et al., 1989). Transformation of *A. thaliana* with *A. tumefaciens* was performed according to the "floral dip method" (Clough and Bent, 1998). As the *bar* gene in the *BniGSL-ALK* expression construct confers phosphinothricin (Basta) resistance to transformed plants, seeds were sowed on soil wetted with a Basta solution and watered with this Basta solution every second day. Plantlets resisting this treatment were transferred to individual pots, watered subsequently without selection agent, and the presence of the transgene was verified by PCR analysis. For this purpose, DNA was extracted from leaves as described by Edwards et al. (1991).

RNA Extraction and Northern Blot Analysis Total RNA was extracted from leaves of 5 wk-old *A. thaliana* plants as described by Logemann et al. (1987). Ten µg of total RNA was fractionated on denaturing 1.5% formaldehyde agarose gels (Sambrook et al., 1989), and equal RNA loading was determined by ethidium bromide staining. The transfer of RNA to Hybond-N nylon membrane (GE Healthcare, Little Chalfont, UK) was done by capillary blotting (Sambrook et al., 1989). A 0.5 kb gel purified (QiaexII Kit, Qiagen, Crawley, UK) fragment generated by PCR amplification on the cloned *BniGSL-ALK* was used as probe. The labeling of the probe was performed with ^{32}P-dCTP (MP Biomedicals, UK) using the DecaLabel DNA Labeling Kit as indicated by the supplier (Fermentas, St. Leon-Rot, Germany). Hybridization was performed in Church buffer at 65ºC overnight (approximately 16 h), and washes were carried out once at 65ºC in 1xSSC, 0.1% SDS for 20 min, and once at 65ºC in 0.1xSSC, 0.1% SDS for 20 min. The Blot was exposed for a day to a BioMAX MS film (Kodak, Hemel Hempstead, UK).

Extraction of Glucosinolates Fully expanded leaves from 5 wk-old *A. thaliana* plants were collected and freeze dried. In the case of the analyzed transgenic line Col-5 (+*BniGSL-ALK*)-4, homozygous plants of the T3 generation were used. Leaf material was ground to a fine powder with a pestle and mortar, and 20 mg were used for the extraction of glucosinolates. The remainder of the extraction process was carried out as previously described (Heaney and Fenwick, 1993).

Analysis of Glucosinolates by HPLC Samples were analysed by high performance liquid chromatography (HPLC) on an Agilent 1200 series instrument equipped with a Phenomenex Luna 3 micron C18(2) (150×2 mm) column. Freeze dried plant tissue was extracted with boiling 80% methanol for 5 min, and the process repeated. At this stage, either 2-propenylglucosinolate or benzylglucosinolate was added as a standard. Combined supernatants were concentrated to dryness with nitrogen gas, and the residue was reconstituted in water (1 ml). A barium/lead acetate (Rossiter et al., 1990) solution (0.1 ml) was added to each sample and allowed to precipitate. After centrifugation, the extract was applied to a DEAE-A25 sephadex column and treated with desulfatase (Rossiter et al., 1990). Desulfoglucosinolates were separated by using a water-acetonitrile gradient (solvent A water, solvent B acetonitrile; 0 – 15 min 25 % B; 15–17 min 70 % B) at a flow rate of 0.2 ml min^{-1} and monitored at 229 nm. Retention times of known standards were used to identify desulfoglucosinolates, and sample peaks were confirmed by LC-MS. For mass spectrometry, an Applied Biosystems QTrap interfaced with APCI source was used. The source temp was set to 475°C, and the MS was run in the Enhanced Mass Spectrum mode scanning in the range 70–500 amu.

Extraction of Glucosinolate Hydrolysis Products Leaves of *A. thaliana* plants (100–200 mg of fresh weight) were crushed and incubated for 10 min at room temperature. The homogenate was extracted with dichloromethane (4 ml), dried with anhydrous $MgSO_4$, centrifuged, and the organic phase concentrated to approximately 200 µl under a flow of nitrogen.

Analysis of Glucosinolate Hydrolysis Profile by GC-MS The dichloromethane extracted glucosinolate hydrolysis products were analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) on a Hewlett-Packard 6890 gas chromatograph linked to a 5973 mass selective detector. Injections were made onto an HP-5MS 5% Phenylmethylsiloxane (30 m×0.25 mm × 0.25 µm) column in the pulsed split (20:1) mode using the following temperature program: inlet temp 225°C; initial temperature 40°C, 5 min; 5°C min^{-1} until 180°C; 10°C min^{-1} until 280°C; hold for 10 min. All compounds were identified by using standards and by their mass fragmentation patterns (Spencer and Daxenbichler, 1980).

Insect Cultures The crucifer specialist aphid *Brevicoryne brassicae* (L.) was maintained at 18°C, with a 16/8 h L/D photoperiod. Aphids were cultured on individual 4 wk-old *Brassica nigra* plants, each plant being enclosed within a perforated bread bag. Aphids were transferred to fresh plants every 1–2 wk.

The aphid parasitoid *Diaeretiella rapae* (McIntosh) (obtained from Rothamsted Research, Harpenden, UK) was maintained at 23°C (16 h light) and 18°C (8 h dark). Standardized cohorts of parasitoids were produced by allowing mated 2–3 d-old adult insects to parasitize mixed

age *B. brassicae* in a Petri dish for approximately 2 h. Parasitized aphids then were transferred to a fresh *B. nigra* plant (unless otherwise stated) on which they continued to feed. Aphid mummies were removed from these plants approximately 8 d later so that on emergence parasitoids were not provided with cues that might influence behavioral responses. Adult male and female parasitoids were kept together, and mating was assumed. Adult parasitoids were provided with a dilute honey solution, and females were used in the bioassays approximately 72 h after emergence.

Aphid Mean Relative Growth Rate Bioassays Young (<24 h-old) *B. brassicae* nymphs were collected from *B. nigra* plants. Each nymph was weighed (MX5, Mettler-Toledo, Greifensee, Switzerland) and transferred individually to a 5 wk-old *A. thaliana* plant. Each plant then was covered with a perforated bread bag and maintained for 5 d at 18°C with a 16/8 h L/D photoperiod. Each aphid then was reweighed and returned to the same plant. The mean relative growth rates (MRGR) were calculated using the following equation:

$$MRGR = (\ln(W_1) - \ln(W_0))/d$$

where W_1=end weight (mg), W_0=start weight (mg) and d= development time (days) (Castle and Berger, 1993).

Comparisons of MRGR values of *B. brassicae* reared either on *A. thaliana* ecotype Col-5 or the Col-5 transgenic line expressing a functional 2-oxoglutarate dependent dioxygenase (*BniGSL-ALK*) were made. Statistical analysis of the MRGR values was completed using unpaired *t*-tests (Genstat 6th Edition).

Olfactometry A Y-tube olfactometer, of the design previously described by Du et al. (1996) was used to record behavioral responses of female *D. rapae* to *A. thaliana* plants infested with *B. brassicae*. A glass Y-tube olfactometer was used, which had 12 mm internal diameter, 100 mm stem and 100 mm arms at 60° angle. Air was pumped through Teflon tubing by a Dymax30 pump (Charles Austen Pumps Ltd., Byfleet, Surrey, UK) through an activated charcoal filter before being regulated by a flowmeter to 800 ml/min. The airflow then was split by a brass T-junction (Swagelok, OH, USA), each flow of 400 ml/min then passing into an airtight glass chamber (T. Westlake — Artistic & Scientific Glassblower, Kidlington, Oxfordshire, UK) into which the volatile source was placed. From the two glass chambers, air flowed through additional Teflon tubing into the arms of the olfactometer via modified glass quick fit sockets. A white cardboard screen was placed around the olfactometer, in order to exclude any visual cues, and lighting was provided by a fluorescent strip held 300 mm above and 100 mm in front of the branches of the olfactometer. A single female *D. rapae* was introduced into the stem of the olfactometer and then given 5 min to make a choice. During this period, if the parasitoid failed to move more than 50 mm up the stem of the olfactometer, it was excluded from the experiment. Parasitoids were recorded as having selected an odor if the insect moved more than 50 mm up one of the arms and remained beyond this point for more than 30 sec. Insects that moved more than 50 mm up the stem but did not select an arm of the olfactometer were recorded as not having selected an odor. After each individual was tested, the position of the odor sources was swapped in order to account for any directional bias by the parasitoids. These criteria are similar to those previously described (Girling et al., 2006; Blande et al., 2007). Glassware was washed with acetone and distilled water and then baked overnight at 200°C.

In the following two experiments, two 5 wk-old (3 wk after pricking out) *A. thaliana* plants were presented as odor sources. Responses of naïve *D. rapae* females to *A. thaliana* ecotypes Ru-0 and Col-5 and the transgenic line Col-5 (+*BniGSL-ALK*)-4 were recorded. In both experiments, responses of naïve female parasitoids were recorded to a *B. brassicae* infested plant and an uninfested plant of the same ecotype/transgenic line. In each experiment, responses were recorded from two ecotypes/transgenic lines in this way. A final comparison was completed by taking the aphid infested plant of each ecotype/transgenic line and presenting these opposite each other. In an initial experiment, naïve *D. rapae* were reared on *B. brassicae*, which in turn were reared on *B. nigra*. *B. brassicae* used to infest Ru-0 or Col-5 plants in this experiment were previously reared on the corresponding ecotype. In a second experiment, naïve *D. rapae* were reared on *B. brassicae*, which in turn were reared on Col-5. *B. brassicae* used to infest Col-5 or transgenic plants in this experiment were previously reared on the corresponding ecotype or transgenic line. For each comparison, plants were replaced after every 10 or 15 replicates. Each plant was either left uninfested or infested with approximately 100 *B. brassicae* (mixed age) for approximately 72 h before completing the bioassay. Naïve female parasitoids, produced as previously described, were used in bioassays.

Chi-square values were used for statistical analysis of numbers of parasitoids responding to each odor source.

Air Entrainments For air entrainments of *B. brassicae* infested *A. thaliana* plants, plants were grown as described above. However, 2 wk after being sown, seedlings were pricked out into the center of 60 mm diam pots. A plastic sheath (5 mm diam) then was carefully placed around each seedling. Seedlings were allowed to continue to grow under the same conditions for a further 3 wk. Approximately 100

mixed age *B. brassicae*, previously reared on the corresponding *A. thaliana* ecotype or transgenic line, were transferred onto a test plant. Three days later, a glass vessel (60 mm diam) was placed around the plant. The vessel was in two parts and was held together with clips, clamping around the plastic sheath that protected the hypocotyl of the plant. Charcoal filtered air was pumped into the vessel at 300 ml min^{-1} through polytetrafluoroethylene (PTFE) tubing (1.6 mm inner diam). Air was drawn out of the vessel at 200 ml min^{-1} through a separate outlet, thereby creating a positive pressure within the vessel and preventing atmospheric air from entering. Air entrainments were completed within a growth chamber set to 12 h light, 150 µmol $m^{-2}s^{-1}$, 65% relative humidity, and constant 20°C. Volatiles were trapped onto Tenax TA (50 mg, mesh 60–80, Supelco, Bellefonte, PA, USA) held in injector liners by plugs of silanized glass wool. Before use, liners were first washed with 2 ml of redistilled diethyl ether before being thermally desorbed at 200ºC under a flow of helium. Tenax TA filled liners were fitted to both the inlet (providing an extra layer of filtration of air entering the vessel) and outlet of each glass vessel. Volatiles were trapped onto the Tenax TA for a period of 4 h. The liners were then thermally desorbed by using an Optic 2 programmable injector (Anatune, Cambridge, UK) connected to a Hewlett Packard 6890 Series gas chromatograph fitted with a Hewlett Packard 5973 mass selective detector (GC-MS). Injector conditions were equilibrated for 30 sec and then ramped from 50 to 200ºC at 16°C s^{-1}. An HP-5MS column (30 m× 0.25 mm × 0.25 µm) was used. The carrier gas was helium (constant flow 30 cm s^{-1}). Initial oven temperature was 50°C held for three min, then ramped to 200ºC at 10ºC min^{-1} and held for a further 2 min.

A comparison of volatile chemicals emitted by the *A. thaliana* ecotype Col-5, the Col-5 (+*BniGSL-ALK*)-4 transgenic line, and the ecotype Ru-0 when infested with approximately 100 mixed age *B. brassicae* (previously reared on the corresponding ecotype or transgenic line) or left uninfested was completed.

Results

Altering the Glucosinolate Profile of the Arabidopsis thaliana Accession Col-5 by Expressing the Dioxygenase BniGSL-ALK A homolog from *Brassica oleracea*, called *BoGSL-ALK*, also has been cloned and characterized previously (Li and Quiros, 2003). We cloned a *Brassica nigra* gene that encodes a putative polypeptide that shows over 90% and about 60% sequence similarity to BoGSL-ALK and AtAOP2, respectively (data not shown). By analogy to *BoGSL-ALK*, we called this gene *BniGSL-ALK*. We expressed it under the control of a CaMV35S promoter in the *A. thaliana* ecotype Col-5, where the predominant glucosinolate in rosette leaves is 4-methylsulfinylbutylglucosinolate (Table 1). As expected, the glucosinolate content of rosette leaves of the transgenic plants expressing *BniGSL-ALK* [called hereafter Col-5 (+*BniGSL-ALK*)] differed qualitatively from the glucosinolate content of the wild-type Col-5 plants. A positive correlation between the transgene expression level and the degree of change of the glucosinolate profile (data not shown) was observed in all characterized independent primary transformants. The glucosinolate profile of rosette leaves of the T3 progeny of the high transgene expression line Col-5 (+*BniGSL-ALK*)-4 is shown in Table 1. It lacked the short-chain methylsulfinylalkylglucosinolates characteristic of the wild-type Col-5 completely, and contained primarily 3-

Table 1 The concentrations of glucosinolates in rosette leaves of five week old Col-5 and Col-5 (+ *BniGSL-ALK*)-4 plants. It was not possible to measure 6-methylsulfinylhexyl-glucosinolate* in the transgenic plant as it co-runs with 3-butenyl glucosinolate. Values are reported as the mean±SE (n=3). 2-Hydroxybut-3-enylglucosinolate is the sum of the *R* and *S* forms

Glucosinolate content (µmolg^{-1})		
	Wild type Col-5	Col-5 (+*BniGSL-ALK*)-4
3-Methylsulfinylpropyl-	1.19±0.13	
4-Methylthiobutyl-	0.63±0.11	
4-Methylsulfinylbutyl-	8.62±1.27	
5-Methylsulfinylpentyl-	0.37±0.04	0.28±0.03
6-Methylsulfinylhexyl-	0.29±0.05	*
7-Methylsulfinylheptyl-	0.21±0.01	0.48±0.07
8-Methylsulfinyloctyl-	1.25±0.05	1.92±0.28
2-hydroxybut-3-enyl-		7.34±1.53
3-butenyl-		9.29±1.99
2-propenyl-		1.41±0.29
Indol-3-ylmethyl-	2.94±0.34	4.64±0.46
4-Methoxy-indol-3-ylmethyl-	2.32±0.25	2.66±0.21
Total	17.83	28.01

butenylglucosinolate and 2-hydroxybut-3-enylglucosinolate and to a lesser extent 2-propenylglucosinolate. Transgenic plants maintained at least wild-type levels of the longer chain methylsulfinylalkylglucosinolates (Table 1). Of the two indolylglucosinolates detected in rosette leaves of both the wild-type Col-5 and the transgenic line, only the levels of indol-3-ylmethylglucosinolate were significantly higher in the latter (Table 1) by a factor of 1.6. Aliphatic glucosinolate and total glucosinolate content in rosette leaves were also higher in the transgenic line compared with wild-type Col-5 (Table 1).

As Col-5 does not possess a functional epithiospecifier protein (ESP) (Lambrix et al., 2001; de Torres Zabala et al., 2005) both the wild-type and the Col-5 (+*BniGSL-ALK*) transgenic lines produce isothiocyanates upon hydrolysis of rosette leaves. Consequently, the hydrolysis profile of the transgenic line Col-5 (+*BniGSL-ALK*)-4 gave the expected 2-propenylisothiocyanate, 3-butenylisothiocyanate, and 5-vinyloxazolidine-2-thione that correspond to the modified glucosinolate profile. The hydrolysis profiles of both wild-type Col-5 and of the transgenic line Col-5 (+*BniGSL-ALK)*)-4 are shown in Fig. 3A and 3B.

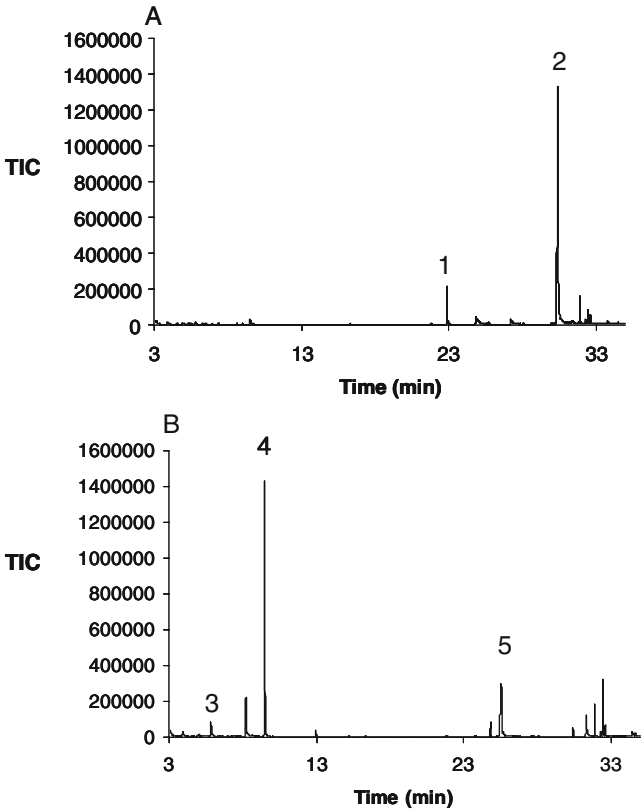

Fig. 3 Glucosinolate hydrolysis products from rosette leaves (5 wk-old) of; **a** wild-type Col-5, peak 1=methylthiobutylisothiocyanate, 2=4-methylsulfinylbutylisothiocyanate **b** transgenic line Col-5 (+*BniGSL-ALK*)-4, peak 3=2-propenylisothiothiocyanate, peak 4=3-butenylisothiocyanate, peak 5=5-vinyloxazolidine-2-thione, analysed by GC-MS. TIC=total ion current

Aphid Mean Relative Growth Rate Bioassays The mean relative growth rate (MRGR) of *B. brassicae* (Fig. 4) was significantly lower when feeding on Col-5 plants transformed with a gene encoding a functional 2-oxoglutarate dependent dioxygenase (*BniGSL-ALK*), compared with wild-type Col-5 plants (t=2.44, P=0.025). This difference corresponds to an increased weight gain of approximately 0.03 mg for aphids feeding on wild-type Col-5 plants compared to aphids feeding on the transgenic Col-5 (+*BniGSL-ALK*)-4 plants during the five-day period of this experiment. It was subsequently recorded that aphids took approximately 0.5 days longer to complete development when feeding on transgenic Col-5 (+*BniGSL-ALK*)-4 plants compared to Col-5 wild-type plants.

Air Entrainments Col-5 plants infested with *B. brassicae* produced no detectable quantities of glucosinolate-derived volatile chemicals (data not shown). However, from air entrainments of plants of the transgenic line Col-5 (+*BniGSL-ALK*)-4 infested with *B. brassicae*, relatively large amounts of 3-butenylisothiocyanate were identified (Fig. 5A, chromatogram a) with a trace of 2-propenylisothiocyanate. In the uninfested line, only a trace amount of 3-butenylisothiocyanate was detected (Fig. 5A, chromatogram b). The *Arabidopsis* ecotype Ru-0 infested with *B. brassicae* produced 2-propenylisothiocyanate (Fig. 5B, chromatogram c), while the uninfested plant produced no detectable isothiocyanate (Fig. 5B, chromatogram d).

Olfactometry Two experiments were carried out to evaluate the response of the parasitoid *D. rapae* towards *B. brassicae* infested and uninfested plants with different

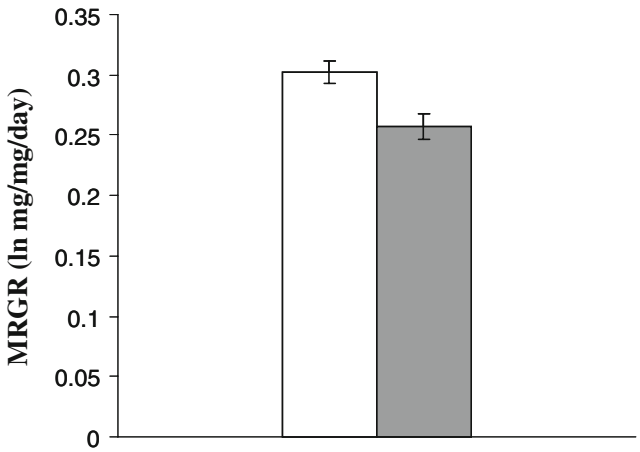

Fig. 4 Mean relative growth rate (MRGR) of individual *Brevicoryne brassicae* confined to single *Arabidopsis thaliana* Col-5 or Col-5 (+*BniGSL-ALK*)-4 plants (Mean±SE, n=15). White bars=Col-5; Grey bars = Col-5 (+*BniGSL-ALK*)-4

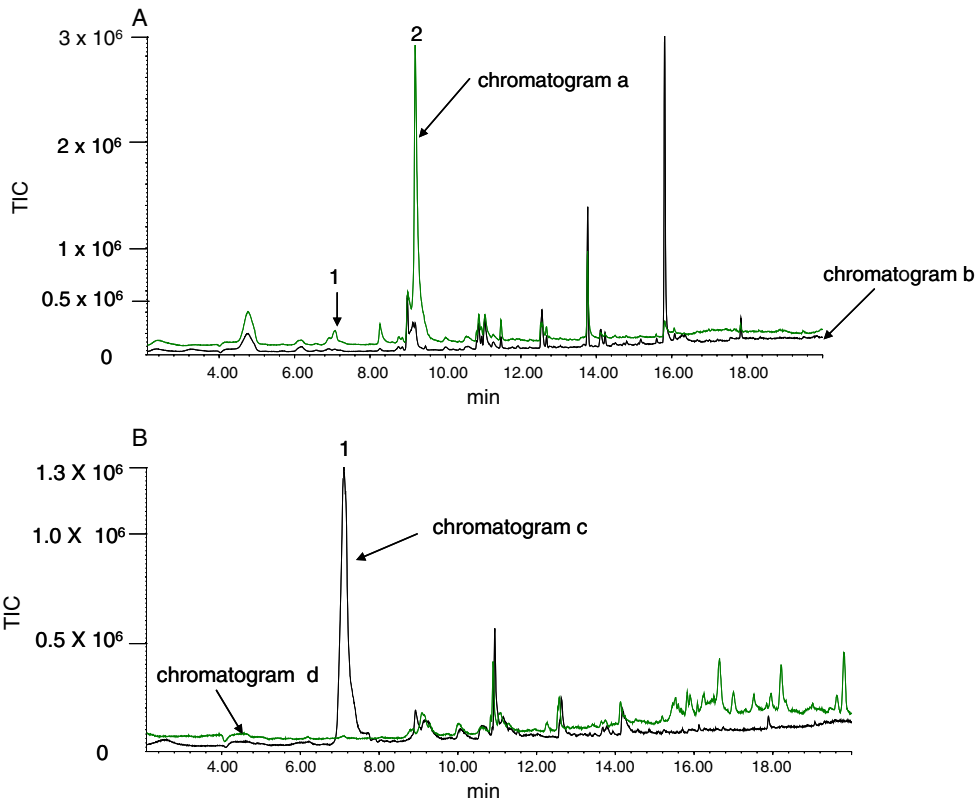

Fig. 5 Air entrainments of (A) transgenic Col-5 (+*BniGSL-ALK*)-4 plants infested with *Brevicoryne brassicae* (trace a) and uninfested (trace b); (B) ecotype Ru-0 infested with *B. brassicae* (trace c) and uninfested (trace d). Plants (5 week old) were exposed to 100 aphids and volatiles were collected 72 h post infestation. Glucosinolate hydrolysis products were analysed by GC-MS. Peak 1=2-propenylisothiothiocyanate, peak 2=3-butenylisothiocyanate. TIC=total ion current

glucosinolate profiles. Results from these experiments indicate that naïve female parasitoids discriminate between aphid infested and uninfested Ru-0 or the transgenic Col-5 (+*BniGSL-ALK*)-4 plants, with *D. rapae* females showing a preference for aphid infested plants. By contrast, *D. rapae* females did not discriminate between aphid infested and uninfested Col-5 plants (Figs. 6A and 6B). When both a *B. brassicae* infested Ru-0 and Col-5 plant were presented as an odor source, parasitoids were again able to discriminate between the odor sources (Fig. 6A). However, naïve *D. rapae* females did not discriminate between aphid infested Col-5 and Col-5 (+*BniGSL-ALK*)-4 plants (Fig. 6B).

Discussion

In order to examine the effect of differing glucosinolate profiles and/or glucosinolate hydrolysis profiles on the crucifer specialist, *B. brassicae*, and its natural enemy, the parasitoid, *D. rapae*, we created transgenic *A. thaliana* lines that overexpress a gene from *B. nigra* encoding a dioxygenase. *B. nigra* accumulates 2-propenylglucosinolate (Cole, 1976) indicating the presence of a functional *AtAOP2* ortholog whose expression in the *A. thaliana* Col-5 background would be expected to result in a glucosinolate profile predominantly composed of 3-butenylglucosinolate. The glucosinolate profile of rosette leaves of transgenic plants that express *BniGSL-ALK,* the *AtAOP2* ortholog we cloned from *B. nigra,* differed from that of the wild-type Col-5 plants and contained the alkenylglucosinolates 3-butenylglucosinolate, 2-hydroxybut-3-enylglucosinolate, and 2-propenylglucosinolate (Table 1). This shows that the *BniGSL-ALK* gene we cloned and transformed into the plant is functional and responsible for converting 4-methylsulfinylbutylglucosinolate and 3-methylsulfinylpropylglucosinolate into the respective alkenylglucosinolates. The transgenic Col-5 (+*BniGSL-ALK*) plants maintain concentrations of the longer chain methylsulfinylalkylglucosinolates (Table 1) suggesting that BniGSL-ALK is unable to catalyze the conversion of these glucosinolates.

The glucosinolate profile changes that we observed in the transgenic Col-5 (+*BniGSL-ALK*)-4 line are in accordance with an earlier report where the authors transformed the Columbia ecotype with *BoGSL-ALK*, a *Brassica oleracea* ortholog of *AtAOP2*, and observed the appearance of the same glucosinolates as we described above (Li and Quiros, 2003). The appearance of 2-hydroxybut-3-enyl glucosinolate in Col-5 (+*BniGSL-ALK*)-4 plants is probably not due to the enzymatic activity of the introduced dioxygenase but to that of another, recently identified dioxygenase (Hansen et al., 2008). The absence of 2-hydroxybut-3-enylglucosinolate in the wild-type Col-5 is

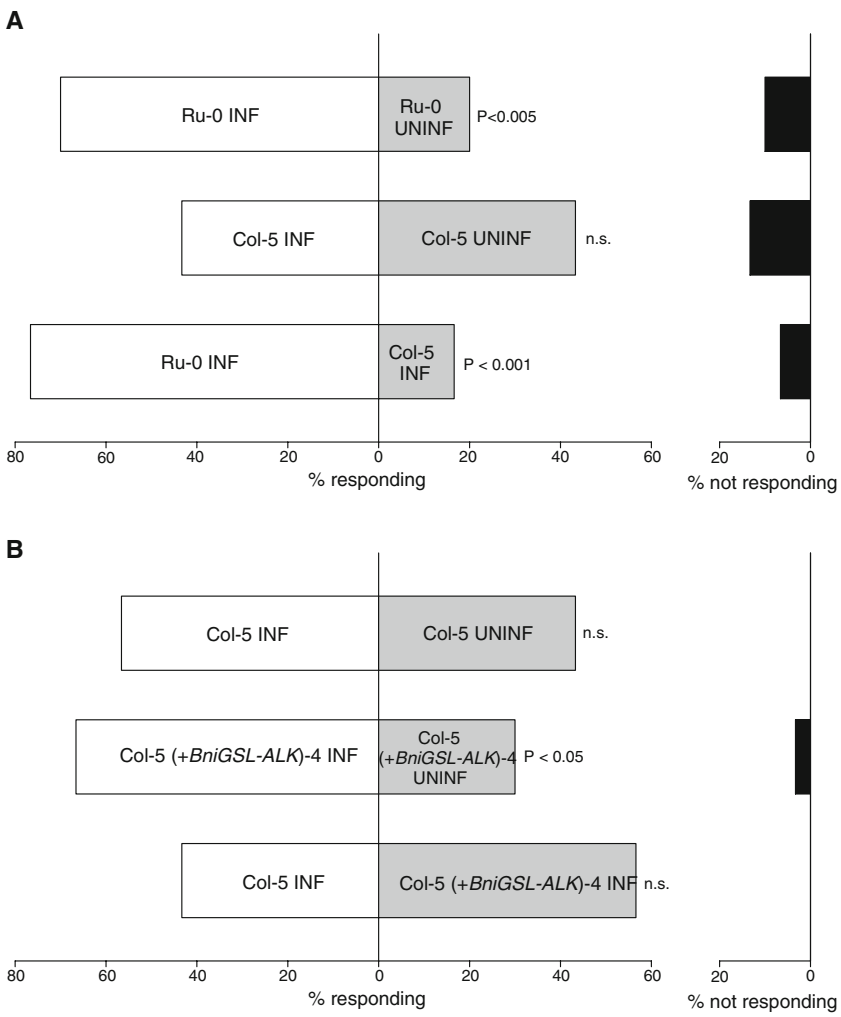

Fig. 6 (A) Responses of naïve *Diaeretiella rapae* females in a Y-tube olfactometer to Ru-0 or Col-5 plants either infested with *Brevicoryne brassicae* or uninfested using *D. rapae* reared on *B. nigra* (n=30); (B) Responses of naïve *Diaeretiella rapae* females in a Y-tube olfactometer to Col-5 or the transgenic line Col-5 (+*BniGSL-ALK*)-4 either infested with *B. brassicae* or uninfested using *D. rapae* reared on Col-5 (n=30); INF=infested, UNINF=uninfested, Col-5 (+*BniGSL-ALK*)=transgenic line expressing *BniGSL-ALK*

due to the absence of substrate (i.e., 3-butenylglucosinolate) as a consequence of the lack of a functional *AtAOP2*.

The fact that indol-3-ylmethylglucosinolate levels were slightly higher in the rosette leaves of the Col-5 (+*BniGSL-ALK*)-4 plants than in the wild-type Col-5 was unexpected. *AtAOP2* is involved in the side chain modifications of aliphatic glucosinolates, and no role in indolylglucosinolate biosynthesis has been reported (Kliebenstein et al., 2001b). Our results are, however, in accordance with a recent report describing a higher total amount of foliar indolylglucosinolate in *A. thaliana* lines that express *BoGSL-ALK* (Wentzell et al., 2007). In addition, we measured a higher total amount of glucosinolates in rosette leaves of the transgenic plants, which was largely due to an increase in aliphatic glucosinolates. In a survey of glucosinolate contents in 39 *A. thaliana* ecotypes, the *GS-AOP* locus (or closely linked loci) was revealed to control 61% of the variation in leaf aliphatic glucosinolates, and ecotypes with *GS-AOP*^{null} presented four times lower aliphatic glucosinolate concentrations than ecotypes with *GS-ALK* (Kliebenstein et al., 2001a). Wentzell et al. (2007) describe a doubling of total foliar aliphatic glucosinolate content in *A. thaliana* lines that express *BoGSL-ALK*. Expressing the *B. nigra* ortholog *BniGSL-ALK* in Col-5 seems to have a similar effect, although the increase in total foliar glucosinolate levels in our transgenic plants was less pronounced than in the above-mentioned study.

The crucifer specialist *B. brassicae* has developed a defense mechanism that allows it to sequester plant-derived glucosinolates (Francis et al., 2001; Kazana et al., 2007), but shows a reduced MRGR when fed on the transgenic Col-5 (+*BniGSL-ALK*)-4 plants compared to the wild-type Col-5. This could be a direct effect of the *BniGSL-ALK* expression, and can be explained by one or a combination of the glucosinolate related changes i.e., change in profile from methylsulfinylalkylglucosinolates to alkenylglucosinolates, increased amounts of indol-3-ylmethylglucosinolate, or increase in the total content of glucosinolates. Previous work with an *A. thaliana* mutant (*atr1D*) that overproduces indolylglucosinolates (Kim et al., 2008) has shown that

these plants are toxic to *Myzus persicae*. However, the increase in indolylglucosinolates in our transgenic plants was small in comparison to that of the *atr1D* mutant. The subsequent changes in hydrolysis products (i.e., production of alkenylisothiocyanates instead of methylsulfinylalkylisothiocyanates, increased amount of hydrolysis products released) could also contribute, although the impact of glucosinolate hydrolysis products produced during attack on these aphids is not clear (Barth and Jander, 2006). However, of significance is the presence of 5-vinyloxazolidine-2-thione in the hydrolysis profile. It is possible that this degradation product has an adverse effect on aphid performance, and this requires further investigation. In addition, plant-aphid interactions are not static, and several, although sometimes conflicting, studies have shown that aphid attacks affect gene expression and glucosinolate levels (Mewis et al., 2005, 2006; de Vos et al., 2007; Kusnierczyk et al., 2007), and such results may contribute to what we observed.

Air entrainment results presented here suggest that the higher volatility of 2-propenylisothiocyanate and 3-butenylisothiocyanate compared with that of 4-methylsulfinylbutylisothiocyanate may be an important determining factor in olfactometry results with naïve *D. rapae* females. Ecotype Ru-0 produced 2-propenylisothiocyanate when infested with *B. brassicae*, but this compound was not detected from uninfested plants. Similarly, the transgenic line Col-5 (+*BniGSL-ALK*)-4 produced 3-butenylisothiocyanate when infested with aphids, while only trace amounts of this compound were detected from uninfested plants. In both cases, naïve *D. rapae* females were able to discriminate between *B. brassicae* infested and uninfested plants. By contrast, for ecotype Col-5 no detectable quantities of glucosinolate-derived volatile chemicals were detected in air entrainments, and naïve female parasitoids were unable to discriminate between aphid infested and uninfested plants. Similarly, it was found (Girling et al., 2006) that naïve *D. rapae* females were unable to discriminate between a Col-*gl* plant infested with *M. persicae* and an uninfested plant. However, parasitoids were able to discriminate between these two odor sources if they were first provided with oviposition experience; thereby suggesting that the volatile profiles of aphid infested and uninfested plants did in fact differ.

By exploiting the wild-type Col-5 and the transgenic line Col-5 (+*BniGSL-ALK*)-4, we were able to record behavioral responses of *D. rapae* females to near isogenic *A. thaliana* plants with contrasting glucosinolate hydrolysis profiles. Interestingly, although more parasitoids responded to aphid infested Col-5 (+*BniGSL-ALK*)-4 plants when presented as odor sources against aphid infested Col-5 plants, the difference was not significant. This suggests that although naïve *D. rapae* females did not discriminate between aphid infested and uninfested Col-5 plants, the odor from the wild-type plant was either weakly attractive or in some way disrupted the behavioral response of the parasitoids to the transgenic plants. By contrast, naïve *D. rapae* females did discriminate between *B. brassicae* infested Ru-0 plants and similarly infested Col-5 plants. This result provides evidence of discrimination between *B. brassicae* infested *A. thaliana* ecotypes by naïve *D. rapae* females, and may also reflect the importance of glucosinolate cues accumulated as the parasitoid emerges from the aphid mummy (Blande et al., 2004). This is because parasitoids responding to Ru-0 plants were reared on *B. brassicae* infested *B. nigra* plants, which like Ru-0 accumulate 2-propenylglucosinolate as the main secondary metabolite. However, although reared on *B. brassicae* infested Col-5 plants, which accumulate 4-methylsulfinylbutylglucosinolate as opposed to the alkenylglucosinolates produced by the transgenic plants, naïve *D. rapae* responded to transgenic but not wild-type plants.

Results presented here indicate that transforming Col-5 plants with *BniGSL-ALK* may increase indirect defense against *B. brassicae* by increasing apparency to its natural enemy, *D. rapae*. Coupled with the reduced MRGRs of *B. brassicae* when feeding on transgenic plants compared with wild-type plants this would suggest that the transformation enhanced both direct and indirect defense against this specialist insect herbivore. This and other work manipulating glucosinolate and hydrolysis profiles by transgenic approaches will lead to a better understanding of the role of secondary metabolites in plant-insect interactions.

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also play a role in regulating plant diversity (Chou 1999), establishment of invasive species (Bousquet-Mélou et al. 2005), and the dynamics in arid environments (Karageorgou et al. 2002).

In the Northern Mediterranean basin, *Pinus halepensis* Miller (Pinaceae, Pinales) is a pioneer and expansionist species that colonizes abandoned agricultural lands characterized by high biodiversity (Roche and Tatoni 1995). Owing to its richness of secondary metabolites (Macchioni et al. 2003), *P. halepensis* may play an important role in plant succession through several processes. For example, secondary compounds (terpenoids and/or phenolic compounds) can affect root symbionts and site quality, by interfering with decomposition, mineralization, and humification (Kainulainen and Holopainen 2002). They can also be involved with interspecific competition phenomena through allelopathic interactions (Rice 1984). Indeed, *P. halepensis* may inhibit seedling establishment of various species in pine stands, suggesting the allelopathic nature of litter, leaf leatchates, and/or root exudates (Fernandez et al. 2006, 2008; Navarro-Cano et al. 2009). Other conifers such as *Pinus sylvestris* L. (Bulut and Demir 2007), *P. densiflora* (Sieb. et Zucc.) (Kato-Noguchi et al. 2009), *Picea abies* (L.) Karst. (Pellissier 1994), or *Picea mariana* (Mill.) (Mallik and Newton 1988) also have allelopathic potential.

Allelopathic potential may be modified by several factors such as the age of the donor plant (Inderjit and Asakawa 2001). Therefore, to understand the role of *P. halepensis* in secondary succession, it is essential to evaluate whether allelochemicals vary in diversity, amount, or function in different plant organs (needles vs. roots), and at different successional stages. For this reason, we analyzed the composition of secondary metabolites in aqueous extracts of roots and needles of *P. halepensis* by searching for polar (fatty acids and phenolic compounds) and less polar compounds (terpenoids) known to be allelopathic (Rice 1984), and we determine whether the chemical diversity of aqueous extracts depends on changes in the age of *P. halepensis* stands.

Methods and Materials

Samples Sites In order to evaluate the variability and allelopathic potential of *P. halepensis* in relation to different stages of secondary succession, three age classes of *P. halepensis* were chosen: (i) Young *P. halepensis* (<15-years-old) called successional stage "Y"—that included meadows colonized by dispersed individuals; (ii) Medium-aged *P. halepensis* (±30-years-old) called successional stage "M"—monospecific or recently closed forest stands, without understory; and (iii) Old *P. halepensis* (>60-years-old) and called successional stage "O"—mature forest with well-developed understory.

Three replicates were collected from each successional stage. Sites were selected along the Southern hillside of the Luberon Mountains in the Natural Regional Park (South of France), on the basis of similar global index (climatic and topoedaphic conditions), by using a model developed by CEMAGREF (Ripert and Vennetier 2002). All sites featured deep agricultural soils (>1 m) of Rendoll in "Soil Taxonomy" (Soil Survey Staff 1999) with no slope and high fertility for *P. halepensis*.

Plant Material Collection and Aqueous Leachates Preparation Needles and roots were collected from five individuals at each site. Needles were harvested from the entire tree crown, and roots were sampled in close proximity to the pines (diam <1.5 m). Just after harvest, needle and root extracts were soaked in water (50 g fw of tissue in 250 ml of distilled water). Extracts were done at room temperature (18°–20°C) and kept in darkness for 24 h. Needle extracts simulated leaf leaching, while root extracts simulated root exudates.

Bioassays. The phytotoxicity of aqueous extracts was tested in previous studies with *Lactuca sativa* seeds, *Linum strictum* (allelopathy, Fernandez et al. 2006), and *Pinus halepensis* (autotoxicity, Fernandez et al. 2008). In these studies, phytotoxicity was analyzed in terms of germination rate and seedling growth (roots and hypocotyles) (Fernandez et al. 2006, 2008), but also in terms of sapling growth (Monnier et al. 2008).

Chemical Analyses

Instrumentation GC-MS analyses were performed on a Hewlett-Packard 6890 GC coupled with an HP5973N Mass Selective Detector. The GC was equipped with an HP-5MS capillary column (30 m×0.25 mm× 0.25 µm—J&W Scientific). Samples were injected with an ALS 7673 Automatic Injector in splitless mode (2 µl for 1 min) for polar compounds (phenolics and fatty acids), and in pulsed splitless mode (5 µl at 25 psi for 1 min) for less polar compounds (terpenoids). Purge flow was set to 50 ml/min after 1 min, except for qualitative studies of polar compounds, for which 30 ml/min were used. Helium (99.995%) was used as carrier gas. A constant flow of 1 ml/min was maintained throughout the runs. Three different oven temperatures were used for qualitative and quantitative studies of polar compounds, and one for less polar compounds. The first program began at 70°C, ramped to 270°C at 5°C/min, and remained at this temperature for 10 min. The second program began at 50°C, increased to 220°C at 5°C/min, and remained at this temperature for 6 min. The third program began at 50°C, increased to 160°C at 2°C/min, and remained at this temperature for 5 min. The

injector temperature (250°C) and MSD transfer line heater (280°C) were the same for all injections. The mass spectrometer parameters for EI mode were: ion source, 230°C; MS quadrupole, 150°C; electron energy, 70 eV; Electron Multiplier Energy 1100–1300 V. Data were acquired in scan mode from 40 to 500 amu for qualitative analyses and quantitative analyses of less polar compounds, and in SIM mode for quantitative analyses of polar compounds.

Chemicals HPLC grade methylene chloride, ethyl acetate, cyclohexane, and acetonitrile were obtained from SDS (Peypin, France). HPLC grade water was used for extractions and a Milli Q system was used for analytical procedures. N, O−bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was used as the derivatizing reagent for qualitative studies with polar compounds. Methylation reagent (methyle iodure, 99.5% purity), internal standard (3-chloroanisole, >97% purity or undecane, 99% purity), HPLC grade methanol, phenolics, fatty acids, phase transfer catalysts (tetrahexylammonium bromure—THAB, > 99% purity), and tri-n-butylmethylphosphonium polymer bound (1.4 mmol Cl^-/g resin—TBMP) were used for quantitative analyses. Analytical grade sodium chloride and potassium dihydrogenophosphate were provided by Prolabo (VWR, France).

Qualitative Analyses

Polar Compounds Qualitative studies of *P. halepensis* fatty (di)acids and phenolics of root and needle extracts were carried out with three randomly-sampled extracts (10%) mixed for each analysis.

The mixed solution was extracted×3 with ethyl acetate (25 ml). The resulting three fractions were pooled, concentrated to dryness, and suspended in methylene chloride (1 ml). The procedure was repeated twice, and the combined solutions were evaporated to dryness by using a stream of helium to remove residual water.

Acetonitrile (200 µl) and BSTFA+1%TMCS (200 µl) were added to the residue. The resulting solution was incubated for 1 h at 70°C, cooled, filtered, and analyzed by GC-MS to produce the trimethylsilyl derivatives.

Less Polar Compounds Aqueous extracts (10 ml of a 10% solution of each extract) were mixed with cyclohexane (1 ml) containing undecane (2 mg/ml) for 1 h. After phase separation, the organic layers were injected onto the GC-MS.

Compound identification was done by comparison of MS spectra to those of reference standards. Database searches in the HP mass spectral libraries were conducted for unidentified components. Retention indexes of compounds were determined relative to Wisconsin Diesel Range Hydrocarbons injection (Interchim, Montlucon, France) and confirmed by comparison with those expected in literature (Adams 1989).

Quantitative Analyses

Polar Compounds Quantitative analyses were performed on needle and root extracts of young, medium, and old pines using a method adapted from Fiamegos et al. (2004). An extraction-derivatization technique was used for phenolics via Phase Transfer Catalysis (PTC). The methylation and extraction methodology was improved to fit phenolics and fatty acids and diacids. The method was tested for a small number of compounds (2 or 3) from each chemical family: fatty acids (palmitic and stearic acids), fatty diacids (succinic and azelaic acids), simple phenols (catechol and pyrogallol), acetophenones (acetovanillone and acetosyringone), phenolic acids (4-hydroxybenzoic, protocatechuic, and gallic acid), and cinnamic acids (*p*-coumaric, caffeic, and sinapic acids). These compounds were selected based on their occurrence in *P. halepensis* (qualitative analysis) or ability to be methylated under these conditions. The quantified derivatives may include several allelochemicals because of the methylation process.

Stock solutions (1 mg/ml) of each compound were prepared by dissolving pure standards (25 mg) in deionized water (25 ml). Methanol/water (1:1 *v*/*v*) was used for less soluble compounds. THAB in dichloromethane (0.1 M; 217.3 mg in 5 ml) and the internal standard were dissolved in dichloromethane.

The procedure was carried out in 35 ml-Pyrex® tubes equipped with PTFE screw caps. Each individual extract (10 ml) was added with stirring to the following solution: KH_2PO_4 buffer (500 µl of a 1 M solution; pH 8.0), TBMP (50 mg), THAB in dichloromethane (100 µl of a 0.1 M solution), internal standard in dichloromethane (50 µl of a 100 µg/ml solution), dichloromethane (850 µl), and methyle iodure (100 µl). Tubes were sealed and heated (80°C) for 1 h to allow methylation. The solutions were cooled, saturated with NaCl, and vigorously shaken. After separation, the organic layer was removed, filtered through a filter syringe (0.45 µm), and analyzed by GC-MS.

The same procedure was used to methylate standards for calibration curves (constructed by taking the ratio of external variable standard to internal constant standard).

Less Polar Compounds An identical procedure was used for less polar compounds. Quantitation was relative to undecane.

Table 1 Mean concentration (± standard deviation) of compounds in root and needle extracts of *Pinus halepensis* (ng/ml)

RI	Common name	Roots			Needles					
		Young	Medium	Old	Young	Medium	Old	F	P	Sign
Monoterpenes										
970	α-Pinene*	2.5 (0.4) a	2.1 (0.3) a	2.3 (0.4) a	0.7 (0.1) b	0.7 (0.2) b	0.3 (0.1) b	12.630	0.001	***
982	Thuja-2,4(10)-diene**	5.2 (1.4) a	3.7 (0.5) a	3.1 (0.5) a	0.0 b	0.1 (0.1) b	Tr b	30.030	0.001	***
994	Sabinene**	0.4 (0.1) a	0.2 (0.1) a	0.1 (0.1) a	0.9 (0.1) b	1.1 (0.2) b	1.1 (0.1) b	19.780	0.001	***
996	β-Pinene*	0.1 (0.1) a	0.2 (0.1) a	0.1 (0.1) a	0.3 (0.1) a	0.1 (0.1) a	0.1 (0.1) a	0.730	0.605	ns
1007	β-Myrcene*	0.8 (0.2) a	0.4 (0.1) a	0.5 (0.1) a	1.1 (0.3) b	0.5 (0.1) a	0.5 (0.1) a	3.400	0.008	***
1026	α-Terpinene*	0.2 (0.1) a	Tr a	0.2 (0.1) a	0.8 (0.2) b	0.5 (0.1) b	0.5 (0.1) b	13.130	0.001	***
1033	p-Cymene*	0.6 (0.3) b	0.0 a	0.5 (0.1) b	0.1 (0.1) a	0.5 (0.2) b	0.5 (0.1) b	2.860	0.020	**
1064	γ-Terpinene*	0.2 (0.2) a	0.0 a	0.0 a	0.7 (0.3) b	0.4 (0.2) b	0.5 (0.2) b	3.140	0.001	***
1113	1,3,8-p-Menthatriene**	0.2 (0.2) a	0.0 a	0.0 a	0.0 a	0.2 (0.1) a	0.3 (0.2) a	1.950	0.095	ns
Oxygenated monoterpenes										
1071	trans-p-Menth-2-en-1-ol**	4.6 (1.1) a	3.7 (1.3) a	4.0 (1.4) a	3.9 (1.0) a	13.5 (1.7) b	12.4 (2.3) b	8.440	0.001	***
1076	cis-Linalool oxide**	0.7 (0.4) a	0.5 (0.3) a	0.3 (0.1) a	4.7 (0.6) bc	6.3 (0.8) c	3.0 (0.5) b	42.390	0.001	***
1091	Fenchone**	0.0 a	0.4 (0.2) b	1.1 (0.3) c	0.1 (0.1) a	0.0 a	0.0 a	8.870	0.001	***
1092	trans-Linalool oxide**	17.7 (13.3) b	1.6 (0.5) a	2.2 (0.6) a	8.8 (1.6) b	8.9 (0.9) b	6.2 (0.8) ab	7.760	0.001	***
1100	cis-p-Menth-2-en-1-ol**	4.6 (0.9) ab	2.2 (0.5) a	1.9 (0.4) a	7.5 (1.5) bc	12.6 (1.7) c	11.3 (1.8) c	14.740	0.001	***
1114	Fenchol, endo-**	0.2 (0.2) a	0.3 (0.1) a	1.1 (0.3) a	0.0 a	0.6 (0.6) a	Tr a	2.620	0.09	ns
1114	cis-Sabinene hydrate**	0.3 (0.1) a	0.2 (0.1) a	0.7 (0.2) a	2.4 (0.6) b	1.8 (0.2) b	1.9 (0.2) b	13.270	0.001	***
1136	trans-Pinocarveol**	10.3 (2.7) b	5.6 (1.1) ab	6.0 (0.9) b	5.7 (0.7) b	4.3 (0.6) a	3.7 (0.5) a	2.500	0.037	*
1138	cis-Verbenol*	4.5 (1.1) b	1.3 (0.4) a	4.4 (3.4) ab	0.6 (0.6) a	0.2 (0.1) a	0.2 (0.2) a	7.750	0.001	***
1141	Camphor*	20.4 (5.5) b	43.5 (9.5) c	50.1 (10.3) c	1.7 (0.2) a	3.6 (0.5) a	3.1 (0.5) a	7.750	0.001	***
1143	trans-Verbenol**	86.2 (28.4) b	45.7 (6.8) b	45.9 (6.6) b	5.8 (1.0) a	7.7 (2.5) a	5.0 (1.1) a	37.820	0.001	***
1163	Borneol*	44.2 (7.6) b	36.5 (6.3) b	46.5 (6.1) b	26.5 (2.5) ab	18.9 (3.2) a	27.4 (3.4) ab	5.570	0.001	***
1171	cis-3-Pinanone**	0.8 (0.2) bc	1.3 (0.3) c	1.7 (0.5) c	0.0 a	0.4 (0.2) ab	Tr a	13.070	0.001	***
1175	4-Terpineol*	14.1 (3.2) a	12.0 (3.8) a	25.1 (8.5) a	173.0 (40.3) b	88.0 (11.7) b	93.4 (11.4) b	25.970	0.001	***
1189	α-Terpineol*	7.2 (1.7) a	5.9 (1.0) a	14.2 (3.3) a	73.5 (7.9) bc	155.9 (7.8) c	117.1 (11.9) bc	91.76	0.001	***
1194	Myrtenol**	27.4 (4.9) b	23.1 (2.7) b	31.0 (4.5) c	2.3 (0.3) a	3.6 (0.4) a	2.4 (0.2) a	82.120	0.001	***
1203	Bornyl ou Isobornyl derivative (formate?)**	6.8 (1.9) ab	4.1 (0.7) a	4.0 (0.9) a	7.2 (0.9) ab	8.9 (1.0) b	5.7 (1.2) ab	4.020	0.001	*
1206	Verbenone*	43.2 (13.3) b	36.6 (4.3) b	36.7 (7.0) b	4.6 (0.6) a	9.3 (1.8) a	6.1 (1.0) a	23.770	0.001	***
1218	(E)-2-Caren-4-ol**	2.0 (0.7) bc	0.5 (0.3) a	0.9 (0.1) a	1.4 (0.2) ab	3.4 (0.4) c	2.0 (0.2) bc	10.420	0.001	***
1285	Bornyl acetate or Isobornyl acetate**	18.1 (2.7) c	13.5 (1.9) bc	11.1 (1.6) b	0.5 (0.4) a	0.0 a	0.0 a	28.990	0.001	***
Sesquiterpenes										
1415	β-Caryophyllene*	0.9 (0.2) bc	1.2 (0.3) c	0.4 (0.2) abc	0.6 (0.2) abc	0.3 (0.1) ab	0.0 a	5.320	0.001	***
1449	α-Caryophyllene*	0.1 (0.1) a	0.1 (0.1) a	Tr a	0.1 (0.1) a	0.1 (0.1) a	0.1 (0.1) a	1.160	0.338	ns
1498	α-Muurolene**	1.1 (0.6) ab	0.5 (0.5) a	3.1 (0.8) b	0.5 (0.5) a	0.8 (0.6) ab	0.5 (0.5) a	3.140	0.012	**
Oxygenated sesquiterpenes										
1548	Elemol**	0.0 a	0.0 a	0.0 a	2.6 (1.4) b	22.2 (2.7) b	17.8 (4.0) b	79.990	0.001	***
1577	Caryophyllene oxide*	8.5 (4.9) ab	2.3 (1.3) a	3.2 (0.8) ab	17.8 (9.4) b	18.2 (8.3) b	36.4 (26.3) b	4.640	0.009	**

Table 1 (continued)

RI	Common name	Roots			Needles					
		Young	Medium	Old	Young	Medium	Old	F	P	Sign
1595	Guaiol**	0.4 (0.3) a	14.7 (11.0) a	5.4 (3.5) a	11.8 (2.7) a	10.9 (2.4) a	7.1 (1.5) ba	1.06	0.397	ns
1644	β-Eudesmol**	1.5 (0.6) a	1.5 (0.5) a	2.0 (0.3) a	18.6 (3.1) c	8.5 (1.2) bc	8.3 (1.3) b	28.600	0.001	***
1651	α-Eudesmol**	0.4 (0.3) a	0.5 (0.5) a	0.0 a	8.4 (1.5) b	5.0 (0.7) b	4.6 (0.7) b	48.630	0.001	***
1651	α-Cadinol**	0.4 (0.2) a	0.5 (0.4) a	Tr a	9.0 (1.4) b	5.5 (0.8) b	5.2 (0.8) b	53.210	0.001	***
Fatty acids										
1041	Succinic Acid*	8161.1 (797.0) c	5654.5 (713.8) b	5229.9 (679.1) b	1466.6 (236.5) a	850.0 (34.7) a	985.3 (105.9) a	59.560	0.001	***
1557	Azelaic acid*	17.6 (4.3) a	11.9 (1.2) a	16.3 (2.1) a	51.5 (4.1) b	38.6 (2.4) b	47.5 (5.6) b	31.030	0.001	***
1935	Palmitic acid*	552.5 (73.6) d	336.3 (43.6) bcd	452.9 (46.4) cd	150.5 (26.4) abc	160.8 (20.6) ab	122.6 (19.0) a	9.760	0.001	***
2159	Stearic acid*	226.1 (24.0) bc	233.9 (33.1) bc	315.2 (32.0) c	140.9 (20.5) abc	102.7 (11.3) ab	85.3 (15.4) a	7.010	0.001	***
Phenolics										
1154	Catechol*	92.0 (83.3) b	5.5 (0.6) ab	5.6 (0.8) ab	2.8 (0.3) a	3.0 (0.2) a	3.6 (0.3) a	4.910	0.001	***
1325	Pyrogallol*	392.4 (37.4) c	388.5 (29.7) c	495.2 (79.0) c	156.9 (23.0) b	73.1 (8.7) a	85.9 (13.6) a	35.450	0.001	***
1384	4-Hydroxybenzoic acid*	120.9 (36.5) b	45.0 (9.6) a	55.9 (5.9) ab	340.4 (37.4) c	74.5 (8.7) b	86.9 (13.9) b	25.270	0.001	***
1602	Protocatechuic acid*	3127.1 (728.2) b	1985.7 (165.5) b	1905.1 (195.8) b	1994.3 (171.6) b	602.3 (76.0) a	851.2 (113.0) a	19.630	0.001	***
1731	Gallic acid*	1418.5 (385.8) b	1333.4 (193.7) b	1473.3 (203.6) b	840.7 (130.8) b	167.8 (29.1) a	198.5 (49.3) a	32.970	0.001	***
1576	Acetovanillone*	64.4 (9.4) a	43.3 (3.4) a	50.2 (3.8) a	216.4 (19.5) b	55.9 (4.1) a	65.5 (10.3) a	35.610	0.001	***
1689	Acetosyringone*	3.2 (1.6) b	1.2 (0.5) ab	1.4 (0.5) ab	4.7 (0.9) c	0.4 (0.1) a	0.5 (0.1) ab	10.570	0.001	***
1681	p-Coumaric acid*	905.3 (134.8) b	364.3 (85.6) a	323.0 (71.5) a	891.9 (93.7) b	236.1 (23.0) a	217.5 (26.9) a	19.340	0.001	***
1894	Caffeic acid*	366.7 (52.9) bc	292.3 (67.3) abc	801.2 (256.9) d	781.0 (110.3) d	166.9 (14.1) a	220.3 (35.5) ab	10.500	0.001	***
2035	Sinapic acid*	3.0 (0.6) a	4.5 (1.4) a	7.5 (1.0) b	7.5 (1.2) b	5.3 (0.8) a	6.3 (1.0) ab	3.650	0.005	**
Others										
987	Benzaldehyde*	0.2 (0.1) a	0.0 a	0.0 a	0.1 (0.1) a	0.1 (0.1) a	0.1 (0.1) a	1.350	0.253	ns
1021	o-Methylanisole**	0.6 (0.2) b	0.2 (0.1) ab	0.1 (0.1) a	0.1 (0.1) a	Tr a	Tr a	5.990	0.001	***
1114	Phenylethyl alcohol**	0.0 a	0.0 a	0.0 a	49.0 (9.3) c	22.0 (4.2) b	16.9 (4.4) b	74.930	0.001	***
1246	Ethyl phenylacetate**	0.0 a	0.0 a	0.0 a	4.3 (1.3) b	3.8 (0.6) b	2.2 (0.5) b	5.750	0.001	***
1408	Methyl eugenol**	0.3 (0.2) a	1.1 (0.4) ab	0.1 (0.1) a	1.3 (0.3) bc	3.4 (0.9) cd	5.2 (0.9) d	13.930	0.001	***
1492	Phenylethyl isovalerate**	5.7 (4.0) a	0.8 (0.7) a	1.0 (0.3) a	43.6 (10.9) b	51.5 (10.8) b	32.2 (6.1) b	61.200	0.001	***
1500	Methylisoeugenol**	0.0 ab	0.0 ab	0.0 a	1.6 (0.6) bc	5.0 (2.3) cd	9.7 (2.3) d	19.930	0.001	***
	Total fatty acids	8957.3 (853.0) b	6236.5 (705.4) b	5918.2 (697.6) b	1809.5 (272.4) a	1152.0 (45.1) a	1240.6 (126.2) a	60.13	0.001	***
	Total phenolics	6493.4 (1324.0)b	4463.7 (394.7) b	5118.4 (600.3) b	5236.5 (482.0) b	1385.2 (130.3) a	1736.1 (251.3) a	26.290	0.000	***
	Total monoterpenes	323.6 (69.3) ab	245.1 (31.3) a	295.4 (38.9) a	334.6 (53.4) b	351.9 (23.7) b	304.7 (23.2) a	10.01	0.265	ns
	Total sesquiterpenes	14.2 (6.7) a	22.0 (14.4) a	15.5 (3.7) a	75.4 (15.3) b	74.5 (12.8) b	82.9 (26.9) b	15.550	0.001	***

Tr traces, *RI* Retention index (retention index of fatty acids and phenolics are those of methyl derivatives because of the extraction/derivatization method employed)

*: compared to authentic standards; **: tentatively identified.

Statistical Analyses Variation in chemical composition by organ type and successional stage was analyzed by using a Canonical Analysis of Principal coordinates (CAP). This is a useful analysis of multivariate data by reference to prior hypotheses [here: no effect of pine compartment or age of pine (successional stage) on chemical composition (Anderson and Willis 2003)]. CAP was the most powerful test for compositional differences among assemblages. As factors appeared to be significant from CAP, they were analyzed by using a Principal Component Analysis (PCA). XL stat® (ver. 4.01) was used for this analysis. Finally, differences in the concentration of each compound by age and pine compartment were tested with the *Kruskall-Wallis test* followed by *post hoc NSK test.* Statgraphics® (version 2.1) was used for these statistical analyses. The chemodiversity index of each tree was calculated according to Iason et al., (2005): $D_{chem} = -\sum [c \log(c)]$ where c is the proportional concentration of each chemical compound.

Results

Chemical Composition Aqueous extracts from needles and roots from the different stands of *P. halepensis* showed a complex mixture of at least 59 identified compounds belonging to different functional phytochemical groups (Knudsen and Gershenzon 2006). Nine monoterpenes, 20 oxygenated monoterpenes, 3 sesquiterpenes, 6 oxygenated sesquiterpenes, 4 fatty acids, 10 phenolic compounds, and 7 others (Table 1) were found in mixtures and consisted predominantly of phenolics (50%), fatty acids (44%), monoterpenoids (5%), and sesquiterpenoids (1%).

Effect of Organ and Successional Stage on Leachates We observed a significant effect of organ type (Canonical Analysis of Principal coordinates, F=20.90; P<0.001) and successional stage (CAP, F=2.87, P<0.001) on chemical composition of leachates.

Figure 1 represents a two-dimensional mapping of the Principal Component Analysis. Axis 1 represents 25.7% of the information, and is characterized on the positive side by two oxygenated sesquiterpenes (α-eudesmol, α-cadinol) and α-terpineol (an oxygenated monoterpene). The negative side shows the concentration of total fatty acids, succinic acid, myrtenol (an oxygenated monoterpene), and a monoterpene derivative (bornyl or isobornyl acetate). Axis 2 represents 15.28% of the information, and is characterized on the positive side by oxygenated and non-oxygenated monoterpenes (e.g., *trans*-pinocarveol, ß-myrcene) and a phenolic compound (4-hydroxybenzoic acid). The hierarchical ascending classification distinguishes two main groups according to organ type. The 1st group is situated on the positive side of Axis 1 and includes all needles analyzed. Needles then were characterized by two oxygenated sesquiterpenes and three oxygenated monoterpenes (α-eudesmol, α-cadinol, and α-terpineol). The second group is located on the negative side of the Axis 1 and includes all the roots analyzed. Roots then were characterized by high concentrations of total fatty acids and other compounds (see above) such as succinic acid.

The "needles group" can be subdivided into 2 sub-groups: young needles (with some young roots) occurring on the positive side of Axis 2 had the highest monoterpene concentrations, while the older needles were located on the negative side of Axis 2.

The Chemodiversity Index (D_{chem}; Iason et al. 2005) was calculated with pooled data from all functional groups and shows that needles have a higher chemical diversity than young and medium-aged pine roots and, to a lesser extent old roots (Fig. 2). When the index is calculated for functional groups, needles have a higher diversity index for total sesquiterpenes (D_{chem} mean of 2.38 for needles and 1.19 for roots), while roots have a higher diversity index for monoterpenes (D_{chem} mean of 3.20 for roots and 2.40 for needles; Tukey tests and one-way Anova, P<0.05). For phenolics and fatty acids, young needles have the highest diversity (D_{chem}=2.38).

Discussion

Numerous compounds were observed in aqueous extracts of *Pinus halepensis*. Monoterpenoids (e.g., α-pinene; sabinene; β-pinene; β-myrcene; α-terpinene; *p*-cymene; γ-terpinene, fenchol, camphor, α-terpineol) and sesquiterpenoids (e.g., β-caryophyllene; α-muurolene; guaiol; α-eudesmol) were found in needles, litter, and needle emissions (Ormeño et al. 2007), as well as in *Pinus halepensis* essential oil (Macchioni et al. 2003). Moreover, the diversity index for monoterpenes in *Pinus halepensis* needles is high (1.7 to 3.1) compared to *Pinus ponderosa* or *Pinus sylvestris* needles (0.9 to 1.5 and 0.8 to 1.7, respectivly) (Iason et al. 2005; Thoss and Byers 2006). Several of these simple phenolics found in *P. halepensis* have been found previously (e.g., gallic acid, 4-hydroxybenzoic acid, vanillic acid, syringic acid, and *p*-coumaric acid) (Robles et al. 2003), as well as in other *Pinus* species (Alonso et al. 2002; Cannac et al. 2007). Fatty acids were recently found in the needles and litter of several conifers (Song and Cui 2003).

The influence of organ and successional stage on the chemical composition of leachates confirms the difference between root and needle leachates—an observation that

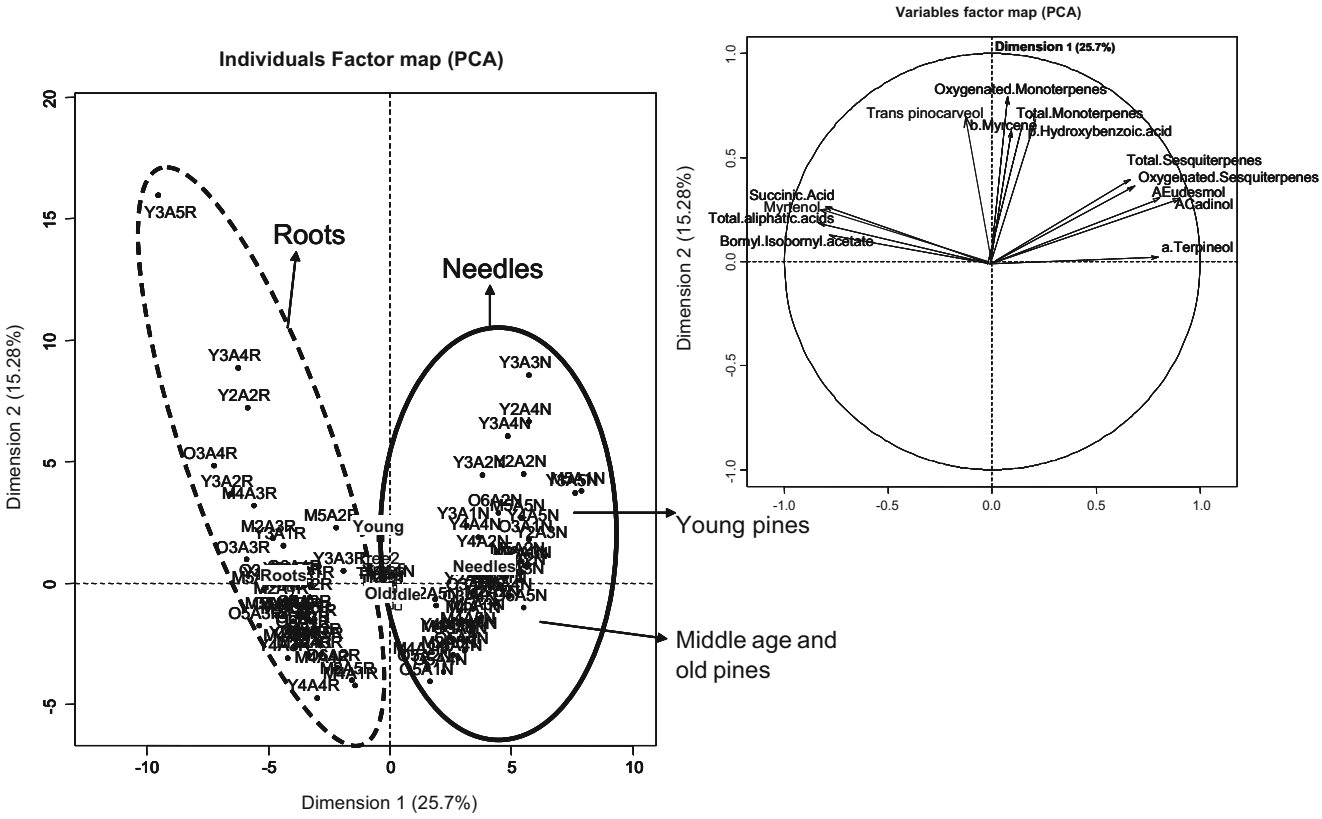

Fig. 1 Two-dimensional mapping of the Principal Component Analysis performed for chemical compounds from different age and organs of *Pinus halepensis* (Y: young <15 years; M: middle age±30 years; and O: old >60 years) and organ (last letter N: needles; R: roots)

parallels the work of Macchioni et al. (2003) with the essential oil of different *Pinus* organs. In general, the concentrations of allelochemicals in conifers decline with age (Liu and An 2003) as does composition and relative amounts of constituents (Julkunen-Tiitto et al. 1996). The chemical variability of *P. halepensis* organs and stage suggest that different organs (i.e., needles or roots) release different chemicals into the environment at different stages and times. Needles show greater variation with the age of the stand. Two sub-groups were identified that had differences between the dominant phytochemical groups in young vs. older pines stands. The highest monoterpene concentration occurred in younger pines needles (Fig. 1) and may be responsible for inhibiting seed germination (Vokou et al.

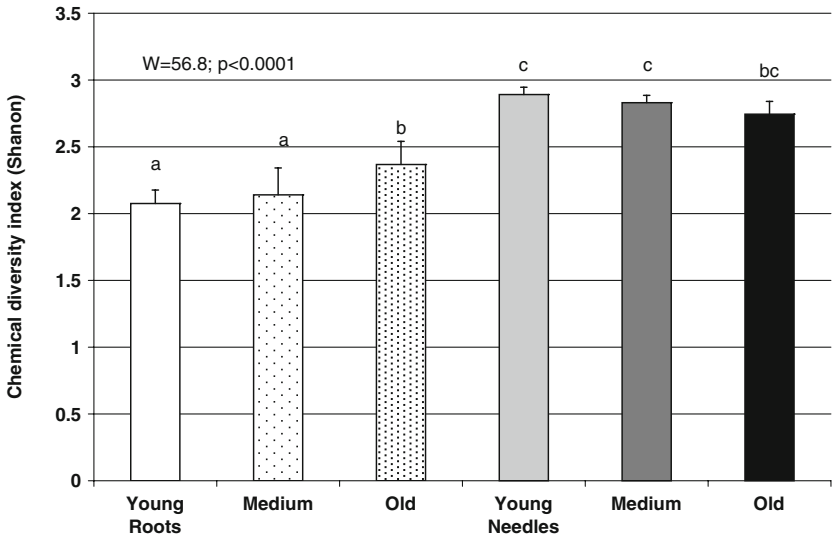

Fig. 2 Chemical diversity of *Pinus halepensis* leachates from different organs and age of calculated according to Iason et al., (2005) (Mean ± SD)

2003) and facilitating the establishment of young pine stands (Fernandez et al. 2006).

The capability of *P. halepensis* to synthesize a rich phenolic mixture during early stages of colonization may confer a competitive advantage in the competition among plants or pathogens. Moreover, the high diversity of compounds in older roots is not surprising given the abundance of competing microbes, insects, and roots of other plants in the same environment. Root monoterpene diversity also may impact tritrophic interactions in soil as these compounds are implicated in indirect defense mechanisms against root feeders. Several studies have now demonstrated that roots can recruit herbivore enemies by releasing chemical cues into soil (Hiltpold and Turlings 2008).

As mentioned previously, secondary metabolites may function in the defense of one plant against another via allelopathic processes. *P. halepensis* leachates, whether from needles or roots, are allelopathic toward some, but not all, plants. Biosensor plants—i.e., *Avena sativa, Lactuca sativa,* and *Lemna minor* (Nektarios et al. 2005)—are inhibited by *P. halepensis* leachates. The leachates also negatively affect wild plants, including: *Festuca arundinacea*, *Cynodon dactylon*, *Linum strictum*, and *Pinus halepensis* (Nektarios et al. 2005; Fernandez et al. 2006, 2008). These findings suggest that the release rate and level of allelochemicals are important factors in the environments in which they occur. Castaldi et al. (2009) showed that *Arbutus unedo* leaves and root extracts were detectable in soil extracts. Similarly, *P. halepensis* extracts may contain a chemical composition similar to those released in nature. Pine needles seem to have higher allelopathic activity in fresh tissue, moderate activity in senescing tissue, and low activity in decaying pine needles (Nektarios et al. 2005). In addition, *P. halepensis* needle litter also negatively affects herbaceous plants (Nektarios et al. 2005), but has no effect on other species—e.g., *Quercus ilex* (Broncano et al. 1998) or *Pistacia lentiscus* (Maestre et al. 2004).

Secondary coumpouds are recalcitrant to decomposition (Vitousek and Reiners 1991). As this constitutes one significant way for allelochemicals to enter the environment (Rice 1984), they can directly influence microbial activity (White 1994), and soil productivity (Bloom and Mallik 2004). *P. halepensis* forests are prone to accumulate relatively thick needle layers below their canopy (Garciaple et al. 1995) and would seem to have the potential to influence biotic interactions in litter (Inderjit and Nilsen 2003) and plant dynamics through allelopathic interactions and mechanical effects on seedling recruitment (Fernandez et al. 2008; Navarro-Cano et al. 2009).

Our previous data showed that young needles and old roots were responsible for most of the allelopathic and autotoxic interactions (Fernandez et al. 2006, 2008; Table 2). The present investigation gives more detail on the principal compounds in both young needles and old roots known to be allelopathic—i.e., sinapic and caffeic acids (phenolic acids) (Table 1) even if both are in low concentrations in *P. halepensis* leachates compared to other compounds (Table 1). Mixtures of phenolic acids and other organic compounds can cause inhibitory effects even though the concentration of individual compounds are well below inhibitory levels (Blum 1996). Caffeic acid (CA) had higher concentrations in both young needles and old roots.

Table 2 Sensitivity of several target species to the highest dose of *Pinus halepensis* extracts from roots and needles and stand age (Y: young < 15 years old, M: medium aged, O: old aged, >60 years old, S: senescent, D: decaying)

Target species	Target type	Roots/ Y	Roots/ M	Roots/ O	Needles/ Y	Needles/ M	Needles/ O	Needles/ S	Needles/ D	References
Lactuca sativa	herbaceous/ target reference	Ge 0 Gr 0	Ge 0 Gr −	Ge 0 Gr−−	Ge 0 Gr +	Ge 0 Gr ++	Ge 0 Gr ++			Fernandez et al. 2006
Linum strictum	herbaceous/ wild species	Ge−− Gr −	Ge − Gr−−	Ge−− Gr−−	Ge−− Gr−−−	Ge − Gr −	Ge−− Gr −			Fernandez et al. 2006
Festuca arundinacea	herbaceous/ wild species					Gr−−−		Gr−−	Gr −	Nektarios et al. 2005
Cynodon dactylon	herbaceous/ wild species					Gr−−−		Gr−−	Gr −	Nektarios et al. 2005
Avena sativa	herbaceous/ wild species					Gr−−−		Gr−−	Gr −	Nektarios et al. 2005
Pinus halepensis	tree/germination stage	Ge − Gr −		Ge−− Gr −	Ge−− Gr 0		Ge − Gr −			Fernandez et al. 2008
Pinus halepensis	tree/ sapling stage				Gr−−					Monnier et al. 2008
Quercus pubescens	tree/ sapling stage				Gr 0					Monnier et al. 2008

GE: GERMINATION; GR: GROWTH; 0: NO EFFECT; −: NEGATIVE EFFECT; +: POSITIVE EFFECT.

This acid is ubiquitous in plants. As with most cinnamic acids, caffeic acid is implicated in many biological interactions (Batish et al. 2008) including allelopathy (Rice 1984) and microbial interactions (Harrison et al. 2007). This compound induces stress in plants, alters physiological and resulting biochemical reactions, and detrimentally impacts plant growth. It is a potent root growth inhibitor (Gallet 1994; Barkosky et al. 2000), and disrupts plant-water relationships and photosynthesis (Barkosky et al. 2000). This phenolic acid changes protease, peroxidase, and polyphenol oxidase activities in root development (Batish et al. 2008) and either interfers with absorption of potassium and phosphorus (Glass 1974), or depolarizes cell membranes in roots (Glass and Dunlop 1974). Caffeic acid has many biological activities and may play a key role in giving *P. halepensis* a competitive advantage over other plants.

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exists about toxic effects (Lamuela-Raventos et al. 2005). In general, the dietary intake of flavonoids traditionally has been regarded as beneficial due to their antioxidant properties; however, supporting data predominantly has come from *in vitro* studies that applied unrealistic high concentrations. Recently, the view has changed, and flavonoids now are less assumed to act as direct antioxidants but rather as inhibitors of pro-oxidant enzymes, such as NADPH oxidases and lipoxygenases, or as chelators of transition metals that mask pro-oxidant actions of reactive nitrogen (NOS) and oxygen species (ROS) (Schewe et al. 2008).

In sharp contrast to the above, (–)-catechin, one of the four isomers of the catechin epimers with *o*-hydroxyl groups on ring B, has been implicated as a chemical weapon of spotted knapweed, *Centaurea maculosa* Lam., which is now classified as a subspecies of *C. stoebe* L., *C. stoebe* ssp. *micranthos* (Gugler) Hayek (Ochsmann 2002). This knapweed species is regarded as an invasive plant in western United States (Callaway et al. 1999; Weir et al. 2003). *Centaurea stoebe* is well known in Europe, as it is one of the more wide-spread knapweed species and is especially common in abandoned dry grasslands (Dostalek and Frantik 2008). Studies that were carried out by Bais and colleagues (Bais et al. 2003; Weir et al. 2003) claimed to provide evidence that (–)-catechin has phytotoxic properties strong enough to support potential contributions of this compound to the invasive success of *C. stoebe* in the western United States. Based on this and similar studies that involved other invasive knapweed species, the novel weapons hypothesis was developed. It states that metabolites of introduced plant species may confer competitive advantages against non-adapted native species (Callaway and Ridenour 2004). The debate concerning the efficacy and ecological relevance of the epimeric mixture of (±)-catechin that is exuded by the roots of *C. stoebe* is ongoing. Besides growth inhibitory effects against some plant species at high concentrations, (±)-catechin also may act as a growth regulator and inducer of systemic resistance at lower concentrations (Weir et al. 2003; Prithiviraj et al. 2007).

We know that diverse abiotic and biotic stresses disturb the redox equilibrium in cells of affected organisms; the thus generated ROS may trigger signal cascades that activate various genes involved in specific response reactions (Wojtaszek 1997; Foyer and Noctor 2005; Gechev et al. 2006; Noctor et al. 2007; Miller et al. 2008). However, if the cell fails to control the levels of ROS, oxidative damage of DNA is inevitable (Halliwell 2006). Iron homeostasis also is vital for the cell. This transition metal is an essential nutrient of plants, yet it often limits growth and, conversely, over-accumulation leads to oxidative stress (Walker and Connolly 2008). In cells, iron is bound to proteins, ferritins, and usually is set free by reductive processes (Laulhere and Briat 1993). Reduced iron can react with H_2O_2 to form hydroxyl radicals ($^{\bullet}OH$), which have enormous potential to damage various organic molecules (Halliwell 2006).

Bais et al. (2003) explored the pro-oxidative effects of (–)-catechin by applying solutions that contained 100 μg ml^{-1} to root tips of *Centaurea stoebe* and *C. maculosa*, the first a known producer of (±)-catechin, and to *Arabidopsis thaliana*, the widely-used model plant in molecular plant physiology. Only the root tips of *C. stoebe* did not show any visible symptoms of induced cell death when ROS production was monitored by imaging generation of dichlorofluorescein (DCF) in the (–)-catechin-treated root tips. Similarly, by using another fluorescent probe, indo-1, the authors showed that levels of Ca^{2+} ions increased in root tips of all tested plants except *C. maculosa* after treatment with a solution of (–)-catechin (100 μg ml^{-1}). Based on this evidence, they proposed that (–)-catechin acts a chemical weapon that contributes to the invasive success of *C. stoebe*. Initially, this study was praised as a fundamental contribution to the respectability of allelopathy (Fitter 2003). Later, however, concerns that the concentrations required for these effects were unrealistic in soil by several orders of magnitude were voiced (Blair et al. 2005, 2006). These concerns recently led to a rejection of the hypothesis that (±)-catechin acts as phytotoxin in allelopathic interactions of *C. stoebe* on the basis of experimental evidence: (1) the concentration of (±)-catechin in soil is too low; (2) (±)-catechin is unstable at pH values above 5, as found in the majority of soils; (3) (±)-catechin is decomposed by extracellular enzymes; and (4) (±)-catechin is only weakly phytotoxic but a strong antioxidant (Blair et al. 2009; Duke et al. 2009a,b). In a series of studies, the originators of the novel weapons hypothesis, concomitantly claimed further support for (±)-catechin as a phytotoxic allelochemical by showing results from experiments that indicated higher susceptibility to (±)-catechin to species in the invaded than in the native range, conditional, however, on the soil type (Inderjit et al. 2008; He et al. 2009; Thorpe et al. 2009).

In this controversy, one further problematic issue has been neglected. Caution needs to be considered when using dichlorofluorescein to detect ROS formation in tissues. The dichlorofluorescein assay is subject to a serious artifact in that it produces what it is purported to measure, i.e., ROS (Bonini et al. 2006). In the cell that possess peroxidase activity, the probe-derived radical may be oxidized, thereby producing further ROS regardless of the initial oxidant (Rota et al. 1999; Bonini et al. 2006). Consequently, the toxic properties of (−)-catechin may have been overestimated by the method applied by Bais et al. (2003). Antioxidative properties of (−)-catechin may contribute to the fact that the producing root tips of *C. stoebe* yield

negative results for ROS production in imaging of DCF-infiltrated tissue. The redox chemistry of (±)-catechin is complex: it has *o*-hydroxyl groups on its ring B, which may either scavenge ROS or chelate free iron ions. Recently, we have shown that pro-oxidative effects of the naphthoquinone juglone, a well-known phytotoxic compound alleged to be the allelopathic agent of the walnut tree (Jose 2002), are strongly enhanced if free iron is chelated directly by juglone (Chobot and Hadacek 2009). In this study, we applied variants of the deoxyribose degradation assay (Halliwell et al. 1987) to explore the redox chemistry of juglone in the presence of free and chelated iron ions [chelating agent ethylenediaminetetraacetic acid (EDTA)] as well as at low and high levels of ROS caused by low and sub-lethal dosages of MeOH.

By assuming that (±)-catechin interacts with ROS, we designed variants of seedling growth assays that resemble different stress levels. This can be achieved simply by adding the organic solvent MeOH (Chobot and Hadacek 2009). MeOH is oxidized to formaldehyde and formate, and during these reactions, ROS are formed (Dobrzynska et al. 1999). Moreover, in attempts to create more complex stress scenarios, all assays were performed simultaneously both in complete dark and in a light/dark regime. If (±)-catechin interacts with signal cascades in the cell, we predicted that similar effects should be visible in a plant as well as in a brine shrimp mortality assay. In a previous study, we demonstrated that nonlinear effects (hormesis) of juglone on seedling growth were due to its excellent capabilities as a redox cycler (Chobot and Hadacek 2009). To explore whether (±)-catechin possesses properties similar to juglone, we decided to contrast the electrochemical potentials of both compounds. This can be measured by cyclic voltammetry, at pH levels characteristic for the cytoplasm and the vacuole, 7.4 and 3.6, respectively. Cyclic voltammetry is one of the less expensive electrochemical methods that are available to study redox chemistry in combination with chemical assays (Firuzi et al. 2005; Chobot et al. 2008).

(±)-Catechin has been shown to be unstable in soils at pH values higher than 5 (Duke et al. 2009a). The pH-dependent instability, oxidation of (±)-catechin (Guyot et al. 1995), may contribute to the variable recovery rates from soils that have been reported in the literature (Blair et al. 2005, 2006; Inderjit et al. 2008; Duke et al. 2009a). To obtain information about (±)-catechin stability in our assay system and in the cell, we used HPLC analysis after 24, 96, and 240 h at pH 3.6 and 7.4, the vacuole and cytoplasm pH, respectively. The deoxyribose degradation assays were performed at pH 7.4.

This combination of analytical, chemical, and biological assays aimed to provide insight into possible mode of actions of (±)-catechin in biotic interactions, not only to contribute to the debate about its acting as a chemical weapon for the invasive *C. stoebe* but also to understand the potential benefits of (±)-catechin accumulation in plant tissues and root exudation.

Materials and Methods

Chemicals Most chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA) unless otherwise stated; water had Milli-Q quality.

Seedling Growth Assays Seeds of black mustard (*Brassica nigra* L.) were obtained from Flora Geissler GmbH (Fisibach, Switzerland) and of white mustard (*Sinapis alba* L.) from B and T World Seeds (Paguignan, France). Solvents were analytical grade. Vapor sterilization of seeds was performed by using commercially available bleach and concentrated HCl (Clerkx et al. 2004). Various variants of the assay were performed; one on agar medium, the other on filter paper. The assays were performed in Petri dishes (9 cm diam, Greiner Bio-One, Kremsmünster, Austria). The agar medium consisted of solidified Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962); (±)-catechin monohydrate (Sigma Aldrich) was dissolved in 2 ml MeOH, and was added to the agar solution after autoclaving. An identical amount of MeOH also was added to the control plates. The surface sterilized seeds (7–9) were transferred to the agar medium with a sterile forceps and actively pressed into the medium to assure maximum contact with the seed surface. Preliminary experiments indicated that effects of the test compounds caused more pronounced effects if this procedure was applied. Filter paper disks (Schleicher and Schuell 520 B 1/2, 60 mm diam) were moistened with 1 ml of a 10%-methanolic aqueous solution containing the test compound in a two-fold serial dilution from 6 to 200 µg ml^{-1}. This measure was taken to prevent crystallization of (±)-catechin (Perry et al. 2005b). The moistened filter papers were placed in 9 cm-Petri dishes. Surface-sterilized seeds (7–9) were transferred to the filter paper with sterilized forceps. Petri dishes were kept sealed for 48 h; then, the sealing tape was removed and MeOH was allowed to evaporate. Water was added to replenish the liquid and to keep the filter paper moistened.

For each concentration, four replicate Petri dishes were prepared. Two were incubated at 25±2°C with a 12 h photoperiod in a temperate greenhouse; the other two were kept completely in dark in the same location. Scoring was performed after 5 days. For practical reasons, of each tested concentration five representative seedlings were chosen for measurement of shoot and root development. The seedlings were photographed with an Olympus

D500 digital camera equipped with a macro lens with 10x magnification. Image analysis was carried out for shoots and roots separately by using Image J 1.36b (Wayne Rasband, NIH, USA, http://rsb.info.nih.gov/ij/). The mean of the pixel counts of the control seedlings was determined as 100% for the quantitative assay (Roberts and Boyce 1972).

Brine Shrimp Assay For each experiment, 0.5 g cysts of *Artemia salina* L., obtained from NovoTemia (JBL GmbH & Co. KG Neuhofen, Germany), were hatched in 25 ml saline aqueous solution (g per 100 ml: 2.3 NaCl, 0.4 Na_2SO_4, 0.07 KCl; pH 8.0±0.2, adjusted with HCl and/or NaOH). For hatching, illumination was performed with a 60 W lamp from a distance of 40 cm for 1 h. The hatched larvae (nauplii) were transferred to an incubator (±25.0°C). One hundred µl of the stock solution (300 µg ml^{-1}) were serially diluted in 96-well microplates, and 50 µl of suspension of 24-h old larvae (6–30 larvae) were added. After 24 h, the wells were scored visually for dead animals (larvae without any movement for at least 10 s) by using an Olympus BHZ 2 stereomicroscope (magnification 8–70 x). Fifty µl of 0.1 M HCl were used to kill all animals. In a second scoring survey, all animals were counted for each well to calculate the number of surviving nauplii for each concentration tested, as required for a quantal biological assay (Roberts and Boyce 1972). $MnCl_2$ served as a positive control. The negative control was the saline solution without test compound. One set of experiments was performed with addition of 5 % MeOH to the stock saline solution of the tested compound; for dilution, only saline solution was used.

Deoxyribose Degradation Assays (H_2O_2/Fe^{3+}/Ascorbic acid) Procedures followed those described earlier for the deoxyribose assay (Halliwell et al. 1987; Aruoma 1994). Here, the ability of the test compound to interact with the reduction of iron (III) by ascorbic acid and the subsequently formed hydroxyl radicals is explored (for a description see Chobot and Hadacek 2009). Briefly, (±)-Catechin monohydrate was dissolved in an aqueous KH_2PO_4/KOH buffer solution (50 mM, pH 7.4); to 125 µl of this solution, 25 µl of a 10.4 mM 2-deoxy-D-ribose solution in the same buffer system and 50 µl of an solution of Fe^{3+} (50 µM) were added. Further, 25 µl 10.0 mM aqueous solution of H_2O_2 and finally 25 µl of 1.0 mM ascorbic acid in buffer were added to start the Fenton reaction. Malonlyldialdehyde (MDA) was determined photometrically at 532 nm after reaction with thiobarbituric acid and subsequent extraction of the red pigment with 1-butanol. The blank contained the full reaction mixture without 2-deoxy-D-ribose. Assays were performed in triplicate.

Deoxyribose Degradation Assays (H_2O_2/Fe^{3+}) This modification was carried out without the addition of ascorbic acid, which was replaced by the same volume of buffer. This variant allows assessing whether the tested compound can reduce iron (III) to iron (II). Scoring was performed after 1 h. The negative control was the H_2O_2/Fe^{3+}/ascorbic acid system mixture that lacked the test compound. The blank contained the full reaction mixture without 2-deoxy-D-ribose.

Deoxyribose Degradation Assays (Fe^{3+}/Ascorbic acid) H_2O_2 was replaced by the same volume of water. Deoxyribose degradation depended strongly on the diffusion of air oxygen into the liquid. This variant allows assessment of the interactions of the tested compound with the H_2O_2 that is formed by reduction of molecular oxygen present in the solution. Consequently, scoring was performed only after 16 h. The blank contained the full reaction mixture without deoxyribose. The negative control was the H_2O_2/Fe^{3+}/ascorbic acid system mixture lacking the test compound.

Deoxyribose Degradation Assays (Fe^{3+}) This modification of the deoxyribose assay was carried out without the addition of H_2O_2 and ascorbic acid, which were replaced by the same volumes of buffer or water. This allows assessment of the reducing capacity of the tested compound of molecular oxygen to H_2O_2 as well as that of iron (III) to iron (II). Scoring was performed after 16 h (diffusion of air oxygen).

Blanks were performed to assess possible reactions between thiobarbituric acid and the tested compound. The values were used to correct the readings obtained in the various modifications of the assay. The blank contained the full reaction mixture without deoxyribose. The negative control was the H_2O_2/Fe^{3+}/ascorbic acid system mixture lacking the test compound (100% MDA).

To the reaction mixture of the deoxyribose degradation assay, 50 µl of Fe^{3+} solution (50 µM) were added. In one series, those 50 µl contained 52 µM EDTA dissolved in buffer, which was premixed with the aqueous $FeCl_3$ solution (1:1 v/v). In the other series, the EDTA solution was replaced by the same volume of the buffer. In the first series, EDTA chelated the iron ions, preventing them from being chelated by the test compound; in the second series, the iron ions were chelated by the test compound.

Cyclic Voltammetry Voltammetric curves were recorded at ambient temperature in a three-electrode µAutolab PGSTAT type III system (EcoChemie Inc., The Netherlands). The working electrode was a glassy carbon electrode (3 mm diam), Ag/AgCl (saturated KCl) was used as a reference electrode, and platinum wire as a counter electrode. The

glassy carbon electrode was cleaned with MeOH and water, and polished before every measurement. The effective scan rate of the CV was 50 mV s^{-1}. The scan potential was from −250 to +1200 mV for (±)-catechin at pH 3.6 and 7.4, for juglone from −400 to +1200 mV at pH 7.4 and from −400 to +1350 mV at pH 3.6. Both substances were dissolved in degassed water. The concentration was 1 mM. The solution for analyses was prepared by mixing 1 ml of the water solution with 9 ml of the degassed buffer. We used phosphate buffer pH 7.4 or acetate buffer pH 3.6. The ionic strength of the buffers was 0.22 M. The ionic strength of the acetate buffer was adjusted by K_2SO_4. The electrolytes were degassed by argon for 10 min, and measurements were carried out under argon atmosphere. Juglone was obtained from Fluka (Buchs, Switzerland).

HPLC Analysis (±)-Catechin monohydrate (Sigma Aldrich, St. Louis, MO, USA) was dissolved in an aqueous KH_2PO_4/K_2HPO_4 buffer solution (0.1 M, pH 7.4) and CH_3COONa/CH_3COOH (0.1 M, pH 3.6) to give a 1 mM solution of (±)-catechin. The HPLC System was a Dionex Summit equipped with a photodiode array detector (PDA) and a Famos autosampler. The column was a Phenomenex Synergi Max C12, 150×2 mm, 5 µm particle size. The column oven was adjusted to 40°C, and the flow rate was 0.2 ml min^{-1}. Solvent A was water : MeOH : *o*-phosphoric

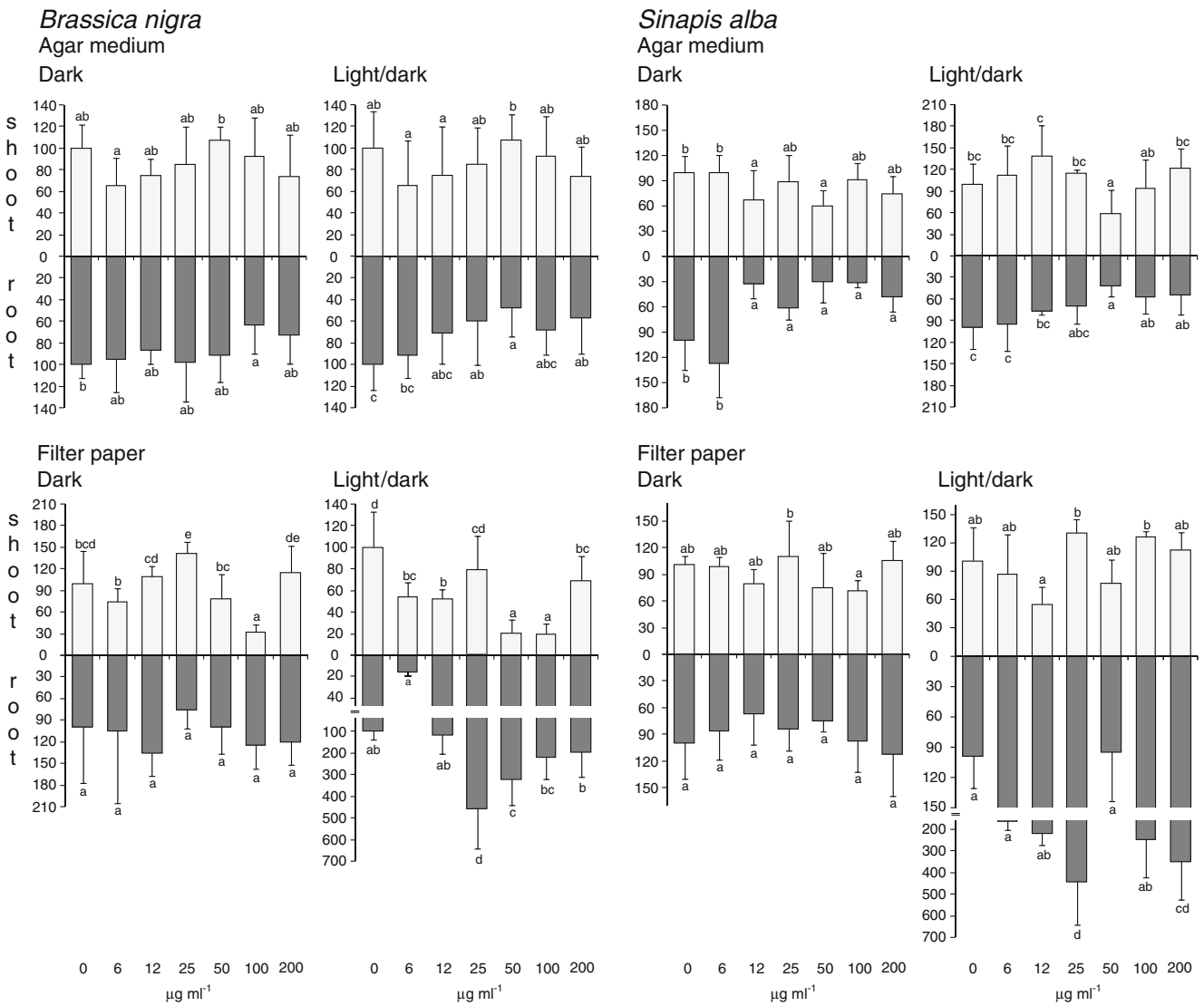


Fig. 1 Effects of (±)-catechin on postgerminative shoot and root growth of *Brassica nigra* and *Sinapis alba* tested on MS agar medium (0.2% MeOH, v/v)) and filter paper (10% MeOH, v/v) after 5 days in temperate greenhouse at 25±2°C in complete dark or a 12 h photoperiod. Growth is expressed as mean of the control treatment; *bars*, means; *error bars*, standard deviation; *N*=5; letters indicate different levels of significance (95% probability, Duncan's multiple range test); 6 (19.48), 12 (38.96), 25 (81.16), 50 (162.33), 100 (324.67), 200 (649.3) µg ml^{-1} (µM)

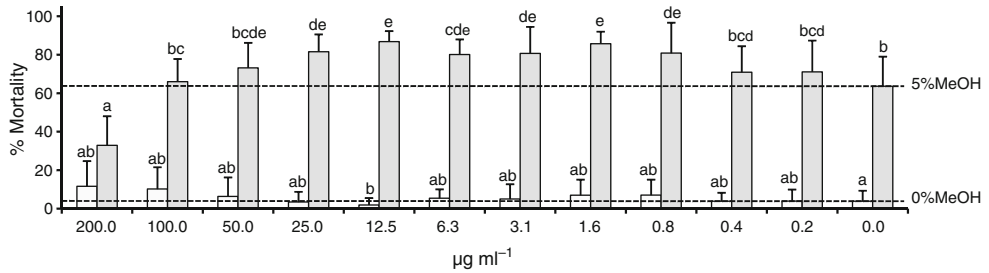


Fig. 2 Effects of (±)-catechin on mortality of 24 h-old nauplii of brine shrimp (*Artemia salina*) in saline aqueous solution after 24 h; one variant with addition of MeOH, the other with 5% (v/v); mortality rate is expressed as percentage of dead larvae; *bars*, means; *error bars*, standard deviation; $N=8$; letters indicate different levels of significance (95% probability, Duncan's multiple range test)); 6 (19.48), 12 (38.96), 25 (81.16), 50 (162.33), 100 (324.67), 200 (649.3) µg ml^{-1} (µM)

acid (9 : 1 : 0.5, v/v/v), solvent B was pure MeOH. The gradient started with 100% of A for 2 min and then linearly changed to 100% of B within 98 min. The final concentration was held further for 10 min. Five µL of (±)-catechin solutions were injected. UV spectra were recorded from 590 to 220 nm. The experiment was repeated twice. One series only, however, is shown.

Statistics Statgraphics Plus 5.0 (Statistical Graphics Corp., Rockville, MD, USA) was used to perform analyses of variance (ANOVA) with Duncan's multiple range tests at a confidence level of 95%.

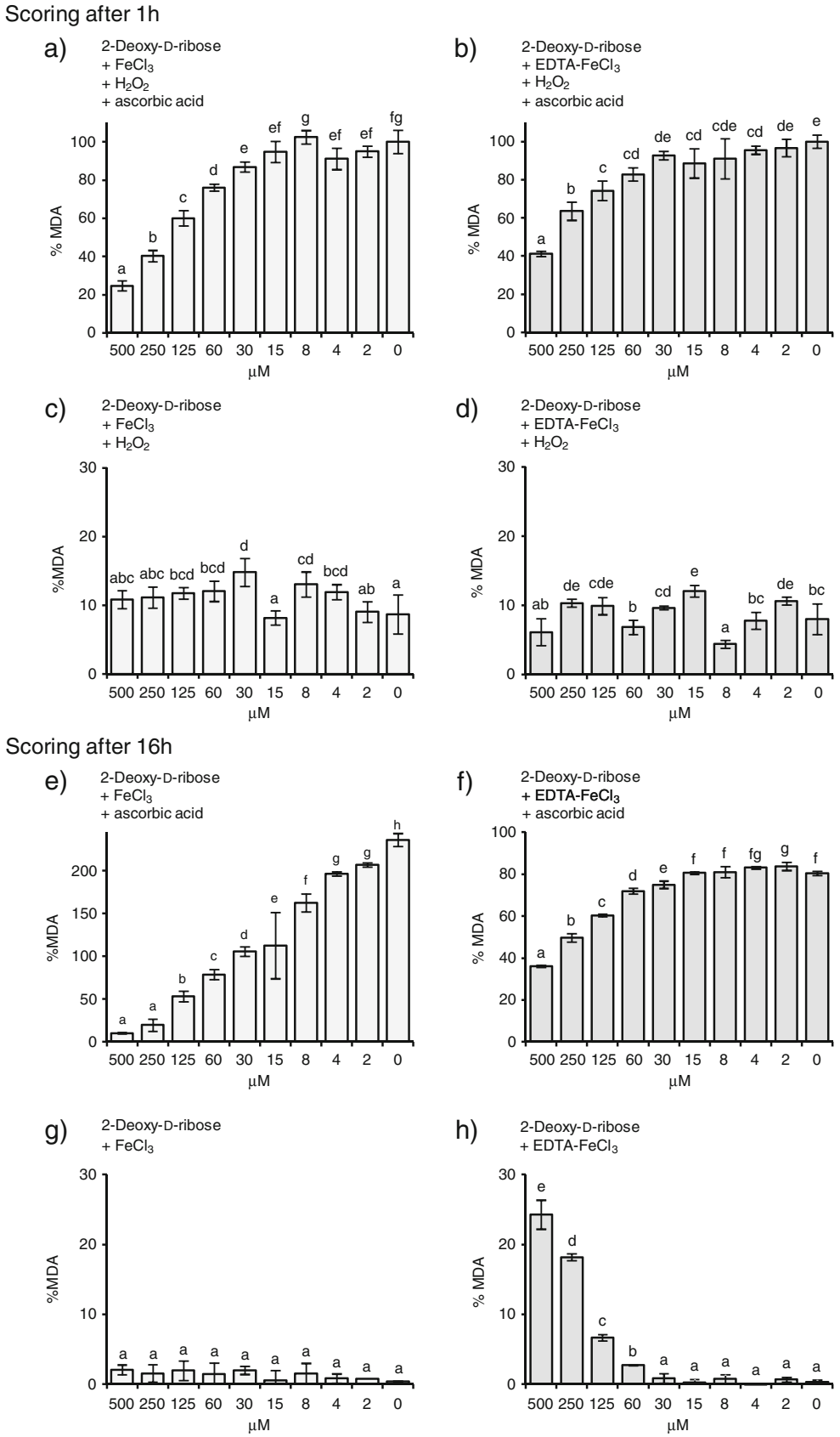
Results

Seedling Growth Figure 1 summarizes the results that were obtained when (±)-catechin was applied in concentrations from 6 to 200 µg ml^{-1} agar medium or filter paper treated with aqueous solution. In both setups, crystallization of (±)-catechin affected the testing of higher concentrations, 100 and 200 µg ml^{-1}, respectively. The two assay designs, as well as the different light regimes, specifically affected the growth of the seedlings of both brassicaeous species. Both mustards were affected similarly by specific conditions, but showed variable susceptibility. The effect that a specific concentration caused on the development of the seedlings ranged from retardation (inhibition) to induction of growth. This depended on the specific assay setup, light regime, or organ (root or shoot). The most notable inhibition was visible in white mustard seedlings, which were grown on agar medium in the dark, starting from 12 µg ml^{-1}. The presence of light slightly attenuated this effect. The black mustard seedlings were less affected. In the filter paper assay, in contrast, the presence of light caused a strong stimulation of root growth in both black mustard and white mustard, but only in the light/dark regime. In many instances, the effects were non-linear, i.e. the highest concentration applied did not cause the strongest effect that was observed within the range of tested concentrations.

Brine Shrimp Assay When 5% MeOH was added to the culture medium, the mortality of the brine shrimp nauplii increased (Fig. 2). In the absence of MeOH, only one concentration (12.5 µg ml^{-1}) significantly decreased mortality rate; however, the difference of means was negligible (4 to 2%). In contrast, when 5 % MeOH was added, the highest concentration tested (200 µg ml^{-1}) attenuated mortality by half (64 to 33%).

Deoxyribose Degradation Assays The results obtained in the variants of the deoxyribose degradation assay are illustrated in Fig. 3. (±)-Catechin was tested in concentrations from 2–500 µM. In the classical setup with H_2O_2 and ascorbic acid (Fig. 3a), (±)-catechin decreased the formation of MDA in a dose-dependent fashion. The effect was more pronounced, when Fe^{3+} was chelated by (±)-catechin (Fig. 3a) than by EDTA (Fig. 3b). This was evident in the difference of the means, as well as in the first concentration that differed significantly from the control. When ascorbic acid was left out, the levels of MDA decreased dramatically (Figs. 3c and d). Although some of the concentrations tested significantly differed from the control, the effects of increasing concentrations of (±)-catechin were negligible compared to the variant when ascorbic acid was present. These rather weak effects did not allow a differentiation of whether Fe^{3+} was chelated by (±)-catechin (Fig. 3c) or EDTA (Fig. 3d).

The additional variants of the assay were scored only after 16 h. Due to absence of H_2O_2 in the reaction mixtures, the observation of effects depended on the diffusion of oxygen from the air into the liquid. The presence of ascorbic acid accelerated the decomposition of deoxyribose into MDA. Again, (±)-catechin attenuated the decomposition process; if Fe^{3+} was chelated directly by (±)-catechin (Fig. 3e), the effect was more pronounced


Scoring after 1h
a)
2-Deoxy-D-ribose
+ FeCl3
+ H2O2
+ ascorbic acid
% MDA
a b c d e ef g ef ef fg
b)
2-Deoxy-D-ribose
+ EDTA-FeCl3
+ H2O2
+ ascorbic acid
% MDA
a b c cd de cd cde cd de e
c)
2-Deoxy-D-ribose
+ FeCl3
+ H2O2
%MDA
abc abc bcd bcd d a cd bcd ab a
d)
2-Deoxy-D-ribose
+ EDTA-FeCl3
+ H2O2
% MDA
ab de cde b cd e a bc de bc
Scoring after 16h
e)
2-Deoxy-D-ribose
+ FeCl3
+ ascorbic acid
%MDA
a a b c d e f g g h
f)
2-Deoxy-D-ribose
+ EDTA-FeCl3
+ ascorbic acid
% MDA
a b c d e f f fg g f
g)
2-Deoxy-D-ribose
+ FeCl3
%MDA
a a a a a a a a a a
h)
2-Deoxy-D-ribose
+ EDTA-FeCl3
% MDA
e d c b a a a a a a
500 250 125 60 30 15 8 4 2 0
µM

◀**Fig. 3** Degradation of 2-D-Deoxyribose quantified by formation of malonlyldialdehyde (MDA) by hydroxyl radicals generated in the Fenton reaction; in the variants (**a**)–(**d**) H_2O_2 and scoring was performed after 1 h; in variants (**e**)–(**h**) no H_2O_2 was added and formation of ROS depended on diffusion of air oxygen into the liquid of the reaction mixture, thus scoring was performed after 16 h; 100 % MDA represents the mean formation of MDA by the standard variant with addition of ascorbic acid, H_2O_2, and $FeCl_3$; *bars*, means; *error bars*, standard deviation; N=3; letters indicate different levels of significance (95% probability, Duncan's multiple range test); 2 (0.58), 4 (1.16), 8 (2.32), 15 (4.35), 30 (8.70), 60 (17.40), 125 (36.25), 250 (72.50), 500 (145) µM (µg ml^{-1}) (±)-catechin

than if Fe^{3+} was chelated by EDTA (Fig. 3f). If ascorbic acid was absent, the levels of MDA generally decreased; in fact, they were the lowest of all variants (Figs. 3g and h). In contrast to the variants where H_2O_2 was present (Fig. 3c and d), in this variant chelation of Fe^{3+} by EDTA (Fig. 3h) caused a significant pro-oxidative effect at the higher concentrations tested. Conversely, when Fe^{3+} was chelated by (±)-catechin, none of the tested (±)-catechin concentrations significantly differed from the control (Fig. 3g).

Cyclic Voltammetry The voltammogram of juglone (Fig. 4) shows one prominent reduction peak (reduction of quinone) and two prominent oxidation peaks (back oxidation of ring B and irreversible oxidation of ring A). The unusual high potential of the oxidation of the hydroxyl group located on ring A is caused by the strong electronegative effect of the adjacent carbonyl function on ring B, which affects the density of the electrons on ring A. The irreversibility of the oxidation of the hydroxyl group on ring A is probably caused by undetectable follow-up reactions of the semiquinone. A comparison of the voltammograms measured at pH 7.4 (cytoplasm) and 3.6 (vacuole) showed that the peak maxima shift to more positive potentials (for B from −0.18 to 0.02 V, for A from 1.01 to 1.21 V, for B' from −0.22 to 0.03 V). At lower pH, the irreversibility of the oxidation of ring A is maintained.

The cyclic voltammogram of (±)-catechin (Fig. 4) is similar to juglone. It shows a quasi-reversible oxidation of the catechol part of the molecule (ring B) and oxidation of resorcinol part of the molecule (ring A). The latter oxidation is not reversible, probably due to follow-up reactions. Similar to juglone, a comparison of the voltammograms measured at pH 7.4 (cytoplasm) and 3.6 (vacuole) showed that the peak maxima shift to more positive potentials (for B from 0.24 to 0.48 V, for A from 0.65 to 0.84 V, for B' from 0.12 to 0.28 V, respectively).

Stability of (±)-Catechin in Aqueous Solutions at Variable pH Figure 5 illustrates the obtained results. At pH 3.6, (±)-catechin remained stable in solution even after 240 h (Fig. 5a). Peak #3 is an impurity or decomposition product that was already present in the commercial sample. At pH 7.4, the first decomposition products (e.g., #1, #4, and #7) were visible. The solution started to have a yellowish hue. After 96 h, several decomposition products were detectable. The color of the solution was now yellow (Fig. 3c). The last analysis, after 240 h, indicated that some decomposition peaks had undergone further reactions because their height decreased (e.g., #1 and #7). A comparative analysis of the UV spectra of the decomposition products revealed that peaks eluting later than 30 min were characterized by UV spectra with broad absorbance maxima above 300 nm (#5–#7) in contrast to those eluting earlier (Fig. 5b).

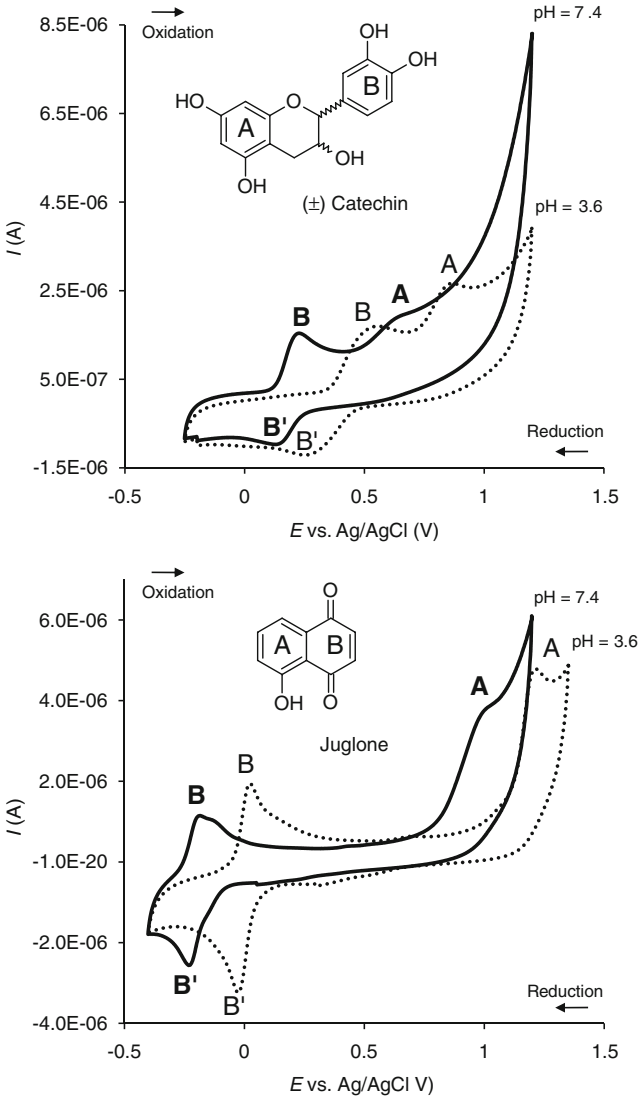


Fig. 4 Cyclic voltammograms of the flavan-3-ol (±)-catechin and the naphthoquinone juglone at pH 7.4 (cytoplasm) and 3.6 (vacuole); letters indicate peaks of oxidation and reduction and correspond to nomenclature of the phenol and quinone ring system

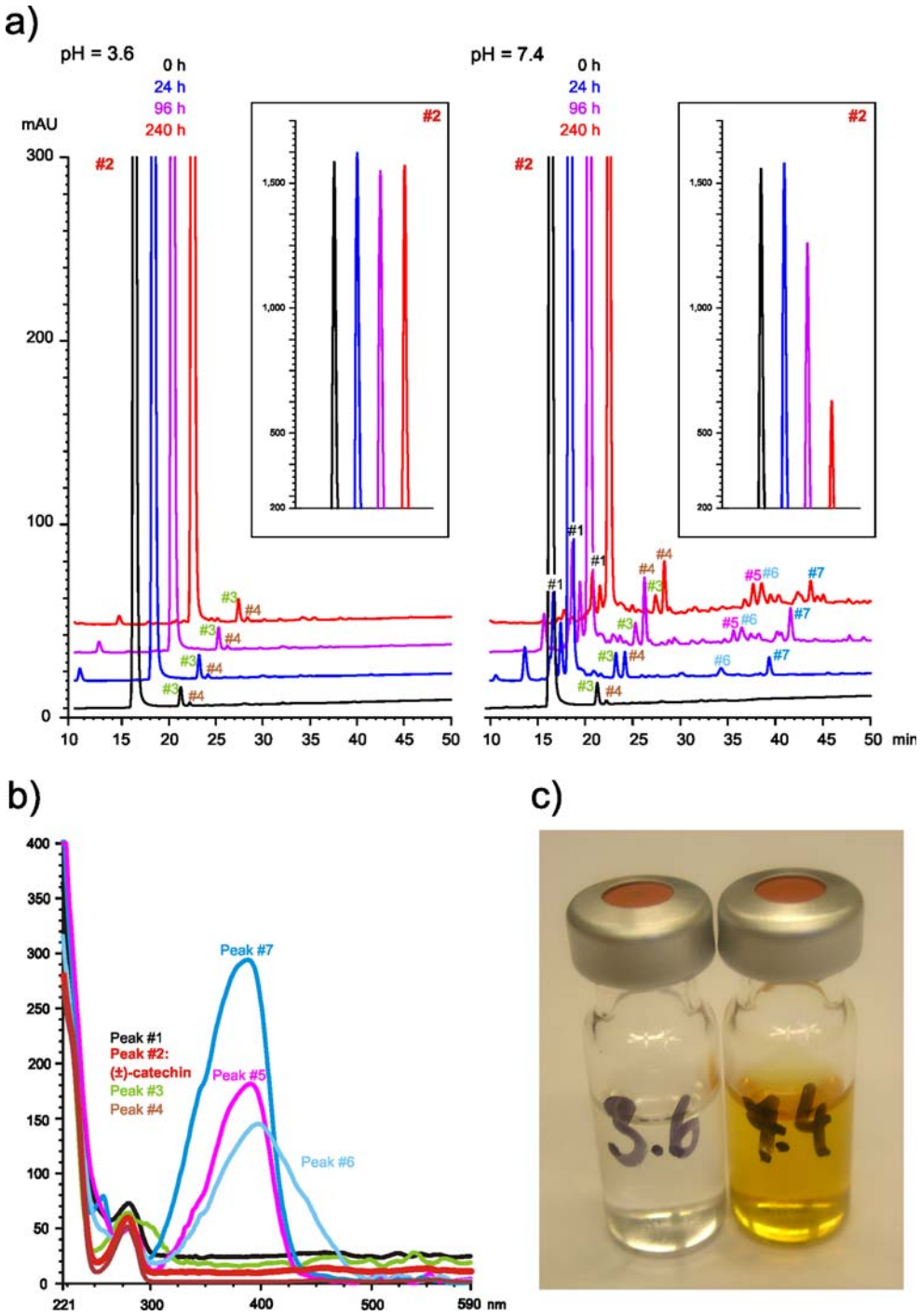


Fig. 5 Stability of (±)-catechin in aqueous solutions: (**a**) HPLC analyses of (±)-catechin at pH=3.6 and pH=7.4 at 0, 24, 96 and 240 h; (**b**) UV spectra of indicated peaks #1–#7, #2=(±)-catechin; (**c**) change of color in solution at pH=7.4 after 96 h compared to pH=3.6

Discussion

Juglone is a well known for its phytotoxic activity (Szabo 1999; Reigosa and Pazos-Malvido 2007), and is a candidate compound for the allelopathy of the walnut tree (Jose 2002). It offers itself, thus, as positive control for the evaluation of phytotoxic effects of (±)-catechin. (±)-Catechin, however, inhibited seedling growth less efficiently by several orders of magnitude; in all variants of the seedling growth assay, juglone totally inhibited growth at concentrations of 50 and 100 µg ml^{-1}, 0.3, and 0.6 mM, respectively (Chobot and Hadacek 2009). Significant differences to this control growth were observed starting from 6 µg ml^{-1}, 0.04 mM, depending on the setup of the assay. Although (±)-catechin managed to affect the growth at similar low concentrations (6 µg ml^{-1}=0.02 mM), in some variants, it never succeeded in inhibiting seedling growth totally. Juglone is known for its pronounced phytotoxic activities at comparatively low concentrations; it even has been

suggested as a reference for an allelopathy index (Szabo 1999). (±)-Catechin has been shown to cause variable effects on different plant species (Weir et al. 2003; Perry et al. 2005a). In this study, we specifically explored the extent of stress level variation on seedlings. In a previous study with juglone (Chobot and Hadacek 2009), we suggested that milieu-dependent radical scavenging reactions of juglone correlate with the stress-attenuating hormetic effects of juglone under higher stress levels (when levels of free radicals are also high). In this regard, (±)-catechin was an even better candidate for exploration in similar assays because its lower phytotoxicity facilitated monitoring the effect across a wider range of sub-lethal concentrations. However, before the discussion of the effects observed in the biological assays may be extended to ecological implications, the effects of (±)-catechin in the deoxyribose degradation assay merits explanation.

The deoxyribose degradation assay was developed to detect hydroxyl radicals, which are formed in the Fenton reaction (1) (Halliwell et al. 1987; Aruoma 1994).

$$H_2O_2 + Fe^{2+} \longrightarrow {}^{\bullet}OH + Fe^{3+} + OH^- \quad (1)$$

In this assay, ascorbic acid, which always is added as the last of all reagents, reduces iron (III) to iron (II) and starts the Fenton reaction. In case of flavonoids, chelation of iron has been implicated in contributing to the antioxidant activity (Cheng et al. 2003). By adding EDTA to the iron solution, the deoxyribose assay offers the possibility of studying the effect of the test compound when iron is either chelated by itself or by EDTA (Aruoma 1994). The iron chelated by EDTA still participates in the Fenton reaction (compare controls in Fig. 3a and b); for detailed reactions see Chobot and Hadacek (2009). Thus, depending on the setup of the assay, iron gets chelated either by (±)-catechin or EDTA (2).

(2)

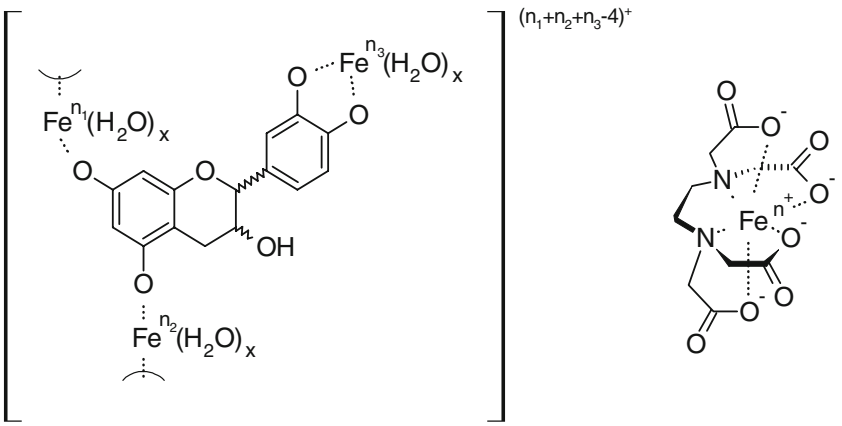
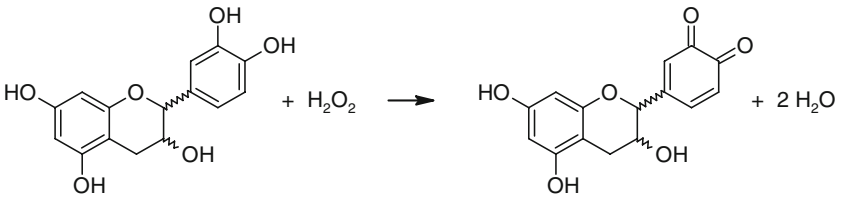


Figure 3a shows the classical variant of the deoxyribose degradation assay. The addition of ascorbic acid creates certain levels of hydroxyl radicals that trigger the formation of MDA. The mean of the control for the classical variant represents 100 % on the y axes of all bar graphs shown in Fig. 3. At lower concentrations (2–8 µM), (±)-catechin chelates the iron ions present in the reaction mixture (2). However, the functionality of the iron is not affected, as the levels of MDA remain unchanged. If iron is chelated by EDTA (Fig. 3b), the levels of MDA also remain unchanged. Ascorbic acid reduces iron (III) to iron (II). With increasing concentrations of (±)-catechin, it starts to react with H_2O_2. The illustrated reactions comprise only a portion of those that are possible. The decreasing levels of MDA in Fig. 3a and b may be explained by the fact that increasing amounts of (±)-catechin reduce H_2O_2 to water by transfer of two electrons (3). Of all ingredients in the reaction mixture, ascorbic acid has the greatest affinity for reducing iron; (±)-catechin does not compare. This is shown by the variants of the deoxyribose degradation assay illustrated in Fig. 3c and d. In this setup, no ascorbic acid was added.

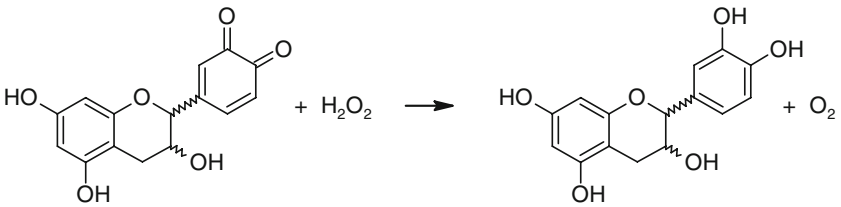
Increasing levels of (±)-catechin did not reduce iron (III), neither if it was chelated by (±)-catechin nor by EDTA. This explains the more or less constants levels of MDA when concentrations of (±)-catechin increased.



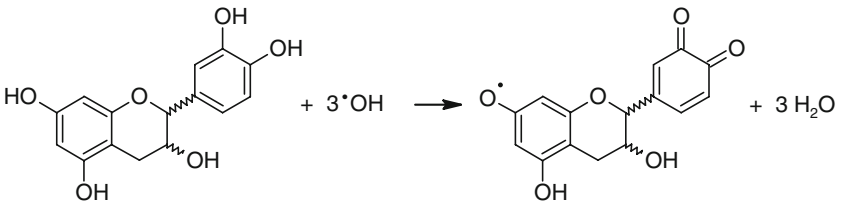
(3)

When (±)-catechin reduces H_2O_2 to water, (±)-catechin gets oxidized to a quinone. This quinone also may react with H_2O_2 and oxidize it to molecular oxygen. During this reaction the quinone is reduced back to (±)-catechin (4).

(4)



Besides the previously illustrated reactions (3, 4), (±)-catechin also may reduce hydroxyl radicals produced by the Fenton reaction to water (5). Such reactions also might provide the precursors for polymerization into proanthocyanidins and condensed tannins (Dixon et al. 2005).

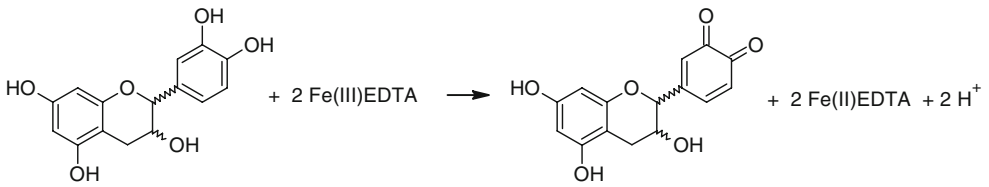


(5)

Slight differences existed between the setup with and without EDTA, which will be explained below.

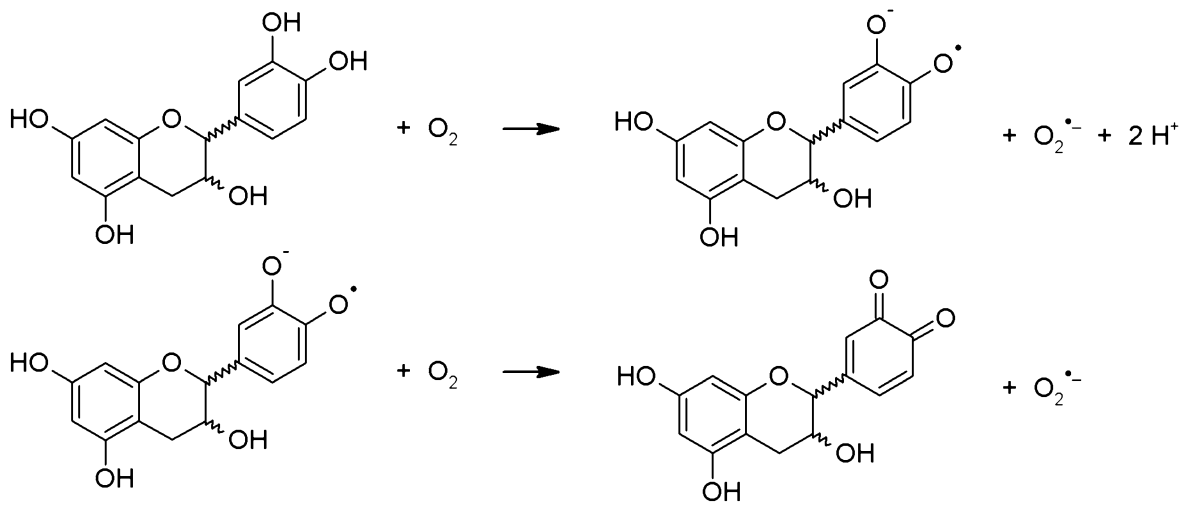
The levels of MDA in Fig. 3a–d were scored after 1 h. In a second series of variants of the deoxyribose degradation assay, no H_2O_2 was added to the reaction mixture. Hydroxyl radical formation depended of the oxidation of diffused oxygen from the air (for detailed reactions see Chobot and Hadacek 2009). Figure 3e and f illustrate that after 16 h, levels of MDA that compare or even exceed that of the standard setup with H_2O_2 could be found in the solution. Similarly, increasing concentrations of (±)-catechin decreased the levels of MDA. Again, the decrease

of MDA was more pronounced in the variant without the addition of the EDTA than in the variant with the addition. The variants of the deoxyribose degradation assay (shown in Fig. 3g and h) revealed that iron chelated by EDTA may be reduced by (±)-catechin (6). Compared to ascorbic acid, this reaction is characterized by a much slower kinetic because the effect only became visible after 16 h.

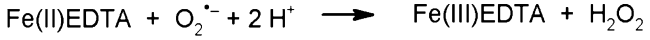


(6)

Simultaneously, (±)-catechin also may reduce molecular oxygen to superoxide anion radical (7), which can be reduced by iron(II) to H_2O_2 (8).



(7)



(8)

H_2O_2 may then further react with iron(II), which becomes increasingly available as rising amounts of (±)-catechin replenish the reduced iron(II) pool. The above outlined reactions represent possible reaction sequences involved in the pro-oxidative effect visible in Fig. 3h. However, if the iron was chelated by (±)-catechin, no reduction of iron in its complex with (±)-catechin occurred. This indicates that the reduction of the iron chelated by (±)-catechin is not allowed thermodynamically in contrast to the EDTA–iron complex. Consequently, the slightly higher levels of MDA in Fig. 3f compared to Fig. 3e may be explained by underlying pro-oxidative effects. In the variants where scoring was performed after 1 h (Fig. 3a vs. Fig. 3b), a similar phenomenon is visible. However, no pro-oxidative effect is visible in Fig. 3d, the variant where ascorbic acid was not added. Although the obtained results allow no unambiguous designation of a specific reaction causing this effect, it is evident that the iron(III) in the iron–EDTA complex is more reactive than in the iron–(±)-catechin complex. This supports the conclusion that chelation of iron adds to the antioxidative effects of (±)-catechin, just as it has been postulated for other flavonoids (Yoshino and Murakami 1998; Mira et al. 2002) and (±)-catechin in dimethyl sulphoxide solution (Bodini et al. 2001).

What implications do these insights have for the interpretation of biological assays of (±)-catechin? In the

seedling development assays we carried out, we observed many non-linear effects, especially in the filter paper assay where seedlings were subjected to high MeOH concentrations in the initial phase. We varied the photoperiod and concentration of the organic solvent MeOH (low in agar medium and high on filter paper) in the test solution of (±)-catechin. Non-linear effects, i.e., non-dosage-dependent stimulation or inhibition of seedling growth, were visible. Such effects also were observed by Weir et al. (2003) and Prithiviraj et al. (2007) who studied the effect of (–)-catechin on a wide range of co-occurring species. The latter authors also attributed this to hormesis, the phenomenon whereby the compound stimulates at low concentrations and inhibits in higher concentrations; it is described by the classical statement of the famous physician Philippus Theophrastus Aureolus Bombastus von Hohenheim, better known as Paracelsus, who lived at the end of the middle ages: "All things are poison and nothing is without poison, only the dose permits something not to be poisonous" [Paracelsus (1538) Third Defensio]. Hormesis is a phenomenon that has long been known but usually has been ignored due to the fact that nobody knew how to explain it (Stebbing 1982). In a recent study, we suggested that milieu-dependent anti- and pro-oxidative activity of juglone may account for hormetic effects caused by this compound under high stress levels (Chobot and Hadacek 2009).

In the present study, we obtained results for (±)-catechin that showed considerable variation depending of the setup of the assay. Identical concentration levels either caused inhibition (agar medium assay) or stimulation of growth (filter paper assay), see light/dark regime in Fig. 1. The stimulation at higher concentrations was visible both in black and white mustard. Thus, our results strongly support a view that was previously voiced by Weir et al. (2003); these authors suggested that (±)-catechin may be a growth regulator. Plants that are exposed to sub-lethal levels of abiotic stress conditions—as in our assay and those carried out in other labs—may exhibit a broad range of morphogenic responses. Those include inhibition of cell elongation, localized stimulation of cell division, and alterations in the cell differentiation status, the stress-induced morphogenic response (SIMR). The similarity in the responses to distinct stresses is postulated as being orchestrated by increased ROS production and altered phytohormone transport (Potters et al. 2007, 2009). The results obtained from our assays resemble such stress-induced morphogenic responses. The idiosyncratic variation of the effects observed at identical concentrations could have been caused by a combination of stress factors that resulted in different levels of morphogenic responses. In contrast to clinical studies, ecological studies are usually less standardized and, as a consequence, lower inter-laboratory comparability exists. In our assays, exact reproducibility of assay conditions—which are nearly impossible to achieve—were replaced by utilizing a low and high level stress scenario to characterize the stress-induced morphogenic response (SIMR) of our test organisms.

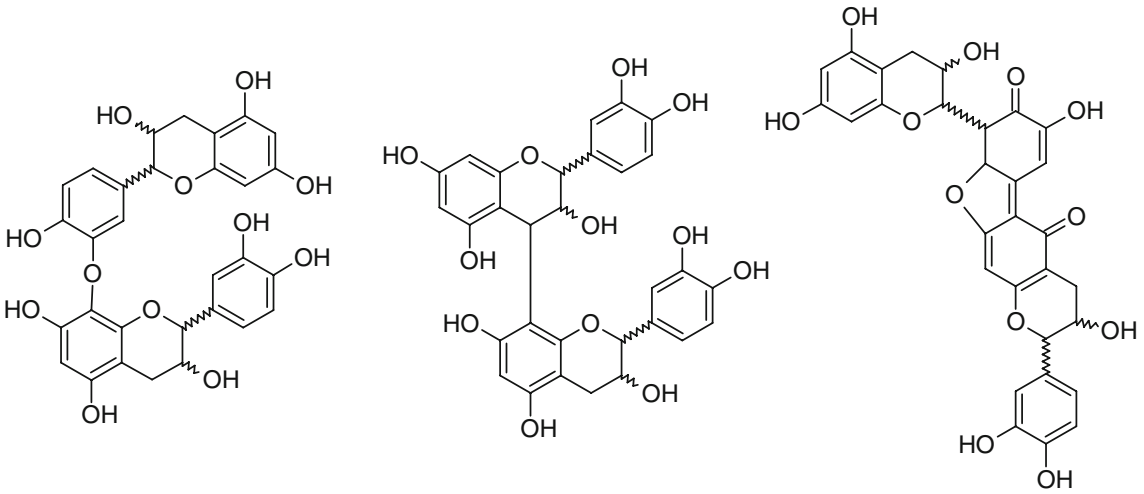
Artemia salina, the brine shrimps, is a crustacean. It susceptibility to (±)-catechin and juglone provides information about potential ecotoxicological effects of these compounds (Nunes et al. 2006). The brine shrimp assay also provides information about intracellular generation of ROS (Matthews 1995; Chobot et al. 2006). The antioxidative effects of (±)-catechin visible in the chemical assays are reflected in the lower mortality rates observed at 5% MeOH concentrations in the saline aqueous solution where concentrations of (±)-catechin increased. Conversely, no change in mortality rates occurred when no MeOH was added to the saline solution. Within the range of tested concentrations, (±)-catechin did not trigger the formation of notable amounts of ROS, as is the case for juglone (IC_{50}= 0.27 mM) (Matthews 1995).

The results from the chemical and biological assays discussed thus far point more to an antioxidative role, (as pointed out by Duke et al. 2009a,b), than to pro-oxidative activity of (±)-catechin (as suggested by Bais et al. 2003). Antioxidative effects were more pronounced when the tested organisms were subjected to higher levels of stress; (±)-catechin reduced these high levels of ROS by scavenging them. Conversely, (±)-catechin may generate ROS, but compared to juglone, which we tested in a similar variant of the deoxyribose assay (Chobot and Hadacek 2009), the pro-oxidative effect is weak. Still, it may account for some of the inhibitory effects of (±)-catechin visible in the seedling growth assays.

Juglone is well known as an efficient redox cycler (Petrova et al. 1990; Bertin et al. 2003). In a recent study, we demonstrated that juglone may either scavenge or generate ROS depending on the milieu (Chobot and Hadacek 2009). The results obtained in our electrochemical experiments provide some evidence that suggest classifying (±)-catechin also as a redox cycler. The cyclic voltammograms of juglone and (±)-catechin show similarities. Both compounds displayed several oxidation peaks, one of which was reversible and accompanied by a corresponding reduction peak, as expected for a redox cycler. At pH 7.4, the peaks showed lower electrode potentials compared to pH 3.6 (Petrova et al. 1990; Martinez et al. 2005). This suggests that compounds such as juglone and (±)-catechin may have evolved to be most reactive in the milieu of the cytoplasm rather than the milieu of the vacuole. Juglone was characterized by reversible peaks at lower redox potentials than (±)-catechin. Juglone, which is a quinone, first gets reduced to trihydroxynaphthalene (Chobot and Hadacek 2009); this compound then is easily oxidized back into the quinone. (±)-Catechin is oxidized first to the

quinone, which then gets reduced to the phenol. In summary, these reactions require lower electrochemical potentials for juglone than for (±)-catechin. In the case of flavonoids, lower electrochemical potentials have been correlated with increased antioxidative properties (Kilmartin and Hsu 2003; Han et al. 2009). Consequently, the results obtained from the electrochemical studies suggest classifying (±)-catechin as a redox cycler, although not so efficient a one as juglone.

In the assessment of potential effects in biotic interactions, (±)-catechin stability is a crucial issue. This flavan-3-ol is a precursor of condensed tannins, which are widespread within plants, and arise from radical polymerization of the semiquinones of (±)-catechin (Dixon et al. 2005). The initial pH in our buffer solutions was 7.4, as in the cytoplasm. HPLC analyses of a 1 mM buffer solution showed that (±)-catechin is decomposed at pH 7.4 but not at pH 3.6. This suggests that (±)-catechin is reactive in the cytoplasm but not in the vacuole. Uptake in roots is evident; brown discoloration occurs when roots are treated with aqueous (±)-catechin solutions (Duke et al. 2009a; unpublished results). HPLC analyses demonstrate that decomposition of (±)-catechin begins already in the aqueous solution. In the rhizosphere, the speed of these reactions may be increased by extracellular enzymes (Duke et al. 2009b). No phenolic acids were detected as were in studies that focus on the decomposition of (±)-catechin in soils (Tharayil et al. 2008). Such phenolic acids, however, may be products of reactions affected by the presence of soil microbes (Pillai and Swarup 2002). The UV-spectra of the peaks of the decomposition products detectable in the HPLC analysis indicate various dimers of (±)-catechin that are formed by radical polymerization of semiquinones (Guyot et al. 1996b) (9).



(9)

The last structure illustrated in this series (9) contains more conjugated double bonds than the other two dimers of (±)-catechin, six vs. three. This chemical characteristic is responsible for the UV spectra that shows absorbance maxima above 300 nm (#5–#7 in Fig. 5b). The HPLC analyses also indicate that only after24 h does (±)-catechin start to decompose. This suggests that our chemical assays were unaffected by the instability of (±)-catechin, but not our biological assays. It is quite possible that such decomposition products could affect the growth of seedlings as well. For instance, decomposition products of (±)-catechin inhibit β-glucosidase activity (Guyot et al. 1996a) and might inhibit other enzymes as well. The tanning effect is well known.

The results from our experiments provide little support for (±)-catechin as a chemical weapon. Rather, they support the recent criticism of this potential function (Blair et al. 2009; Duke et al. 2009a,b): (1) the deoxyribose degradation assay indicated only weak pro-oxidative activity that does not compare favorably to pro-oxidative activities of renowned phytotoxic compounds such as juglone, and suggests that the ROS generating effect of (±)-catechin as shown by imaging of dichlorofluorescein might have been overestimated due to artifact formation; (2) the inhibition of seedling growth reveals more hormesis-like effects, especially under increased stress; (3) the previous hormesis-like effect may be facilitated by the pronounced antioxidative effects of (±)-catechin (Duke et al. 2009a); (4) the

antioxidative properties of (±)-catechin that are evident in the brine shrimp assay and the lack of increased mortality rates also suggest low ecotoxicological potential; and (5) (±)-catechin is stable only at very low pH levels. Consequently, the necessity of proving phytotoxicity in the field, an ultimate proof for allelopathy (Romeo 2000), remains still to be demonstrated for (±)-catechin. The many studies that claim allelopathic activity for (±)-catechin in the field do not provide convincing evidence that it is actually involved in this effect. The observed effects potentially could be caused by different soil chemistry in native and invasive soils that affects the availability of nutrients. Recent cross-continental studies (e.g., Inderjit et al. 2008; He et al. 2009; Thorpe et al. 2009) do not provide any attention to soil chemical characteristics, nor even to pH.

The wide occurrence of (±)-catechin in plants, however, suggests that it might confer benefits, either when accumulated or exuded by roots. Within tissues this benefit might be contributing to stress tolerance as many other phenolic compounds do (Close and McArthur 2002; Grace 2005; Hatier and Gould 2008). In the rhizosphere, (±)-catechin might protect the root tip as follows: Decomposition of soil organic matter (SOM), a process that occurs in soils containing plant litter, especially cellulose, involves transition metal-dependent generation of hydroxyl radicals by the Fenton reaction (Goodell et al. 1997, 2006; Baldrian and Valaskova 2008;). First, exuded (±)-catechin likely chelates iron in a fashion such that its participation in the Fenton reaction is energetically unlikely, and, second, it may scavenge free radicals potentially harmful to the root tips.

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ERRATUM

Pretreatment of Clover Seeds with Nod Factors Improves Growth and Nodulation of *Trifolium pratense*

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The original article unfortunately contained a mistake. The authors regret overlooking and failing to cite a key laboratory study entitled "Nod-factor-treated *Medicago truncatula* roots and seeds show an increased number of nodules when inoculated with a limiting population of *Sinorhizobium meliloti*" by Macchiavelli, R. and Brelles-Mariño, G. (2004) J. Exp. Botany, 55: 2635–40. That paper, a pioneering contribution, showed that not only roots, but also seeds are able to perceive Nod Factors, as evidenced by higher number of nodules in the plant.

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de-novo only after herbivore-damage (Paré and Tumlinson 1999). Among the HIPV, some compounds are emitted immediately upon herbivore damage, while the synthesis of others is truly induced, and it takes a certain time after initial infestation before they are emitted (Turlings et al. 1998). Several genes involved in the biosynthesis of HIPV now have been identified, and this opens the possibility to apply molecular tools to study their role both as attractants for natural enemies and as signaling compounds within and between plant species (Baldwin et al. 2001; Kappers et al. 2005; Paschold et al. 2006; Schnee et al. 2006; Ton et al. 2007; Frost et al. 2008).

Maize has been a model plant since the beginning of the studies on these chemically-mediated tritrophic interactions (e.g., Turlings et al. 1990), and the volatile blends emitted from the aboveground shoot, as well as from the belowground roots, are well characterized (Degen et al. 2004; Köllner et al. 2004; Rasmann and Turlings 2008). Despite detailed knowledge on the chemical composition and the relevance of the entire blend as a host location cue to a range of parasitoid species (Tamò et al. 2006), we still lack an understanding of the relative importance of individual compounds or group of compound to specific parasitoids. Specifically, the importance of minor compounds that are emitted in quantities below the threshold level or compounds which cannot easily be detected by common analytical methods is unknown.

In this study, we used a combination of different fractionation methods (filtering tubes, preparative GC and different solvents) and olfactometer bioassays in order to study the attractiveness of different fractions of a blend of HIPV emitted by maize seedlings infested by *Spodoptera littoralis* (Lepidoptera: Noctuidae) to females of the parasitoid *Cotesia marginiventris* (Hymenoptera: Braconidae). *Cotesia marginiventris* is an important larval parasitoid of *Spodoptera* spp. larvae, which are major pest insects that cause substantial economic damage to maize throughout the Americas. The attraction of *C. marginiventris* females to *Spodoptera*-induced maize volatiles has been investigated in a series of previous studies, which showed clearly that this wasp strongly prefers volatiles emitted by caterpillar-infested maize seedlings over non-infested healthy seedlings (Turlings et al. 1991a, b; 2004). Recent studies, however, indicate that not all compounds emitted by infested seedlings are attractive, and some might even be repellent or mask attractiveness (D'Alessandro and Turlings 2005; D'Alessandro et al. 2006). This was evident from a series of experiments in which we tested attraction of *C. marginiventris* females to volatiles emitted by *Spodoptera*-infested maize seedlings after passing the volatile blend through a selection of adsorbent-containing filter tubes (D'Alessandro and Turlings 2005). Surprisingly, the volatile blend that broke through a filter filled with silica had lost all attractiveness to naïve females, although it still contained at least 70% of the volatiles of the original blend. By contrast, the volatile compounds that were adsorbed by the filter (silica extract) and subsequently extracted with a solvent and applied to filter paper were extremely attractive to *C. marginiventris* females.

Hence, the objective of the current study was to confirm and examine the high attractiveness of the volatiles in the silica extract, as a first step towards characterizing and identifying key volatile compounds or combinations of compounds that are used as foraging cues by *C. marginiventris*. Modifying the release of such key compounds in maize seedlings or applying synthetic versions of these compounds in a maize field could be part of a sustainable and environmentally sound strategy to control *Spodpotera* larvae feeding on maize.

Methods and Materials

Insects and Plants

The caterpillar *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) and the solitary endoparasitoid, *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) were reared as previously described (Turlings et al. 2004). Adult parasitoids were kept in plastic cages in incubators (25±1°C, 16:8 h L/D) and transferred to the laboratory 30 min before the bioassays. Two-4-d-old naive females were used in the bioassays. Plants (*Zea mays*, var. Delprim) were grown in plastic pots (10 cm high, 4 cm diam) with commercial potting soil (Ricoter Aussaaterde, Aarberg, Switzerland) in a climate chamber (25± 2°C, 60% r.h., 16:8 h L/D, and 50,000 lm/m2). Plants used for the volatile collection were 10-12-d-old and had 3 fully developed leaves. The evening before the volatile collection they were infested with 20 s instar *S. littoralis*, which were released in the whorl of the youngest leaf. After infestation, plants were kept under laboratory conditions (25±2°C, 40± 10% r.h., 16:8 h L/D, and 8000 lm/m2), and volatiles were collected on the following day, between 10 A.M. and 4 P.M.

Volatile Collection

Volatiles were collected by passing the herbivore induced volatile blend over a filter tube filled with 25 mg silica (63–200 mesh, 60 Å, Brunschwig, Basel, Switzerland) for 2.5 h and by adsorbing the breakthrough volatiles on a filter tube that contained 25 mg of the highly adsorbent SuperQ (25 mg, 80–100 mesh, Alltech Associates, Inc., Deerfield, IL, USA). Silica is a rather weak adsorbent and used mainly to adsorb polar compounds. Many volatiles break through the silica filters and can be recollected on SuperQ, which is a strong adsorbent and

commonly used to collect a broad range of different volatile compounds (D'Alessandro and Turlings 2005). Volatiles retained by the silica filters were desorbed with 300 µl dichloromethane (Suprasolve, GC-grade, Merck, Darmstadt, Germany) (silica extract). The volatiles adsorbed on the SuperQ filters were extracted with 150 µl dichloromethane (breakthrough). Further details on the volatile collection and volatile filtration are described by D'Alessandro and Turlings (2005). Several samples were pooled in order to obtain a standardized stock solution for each treatment (silica extract and breakthrough), which was stored at −80°C in small vials (Supelco, Amber Vial, 7 ml with solid cap w/PTFE Liner), and used throughout the experiments for all fractionation steps and bioassays.

Fractionation and Analyses of Volatile Blends

The silica extract was separated into several fractions by preparative gas-chromatography (preparative GC). For this purpose, a Hewlett Packard HP 6890 GC with an automated column injection system (HP G1513 A) was either equipped with a non-polar (HP-1 MS, 30 m, 0.25 mm ID, 0.25 µm film thickness; Alltech Associates, Inc, USA) or a polar column (HP-Innowax, 30 m, 0.25 mm ID, 0.25 µm film thickness; Alltech Associates, Inc, USA). Helium at constant pressure (non-polar column: 19.39 psi; polar column: 45.14 psi) was used as carrier gas, and 5 µl of the silica extract were injected in the "on column mode". After injection, the non-polar column temperature was maintained at 40°C for 3.5 min and then increased to 100°C at 8°C/min and subsequently to 200°C at 5°C/min followed by a post-run of 5 min at 250°C. The polar column was also maintained at 40°C for 3.5 min but then increased to 250°C at 8°C/min followed by a post-run of 5 min at 250°C. Fractions were recollected at specific retention times as indicated in Fig. 3 on Pasteur pipettes that contained 20 mg of SuperQ. The precise retention time for each fraction was calculated by installing the outlet of the GC column to a flame ionization detector (FID) prior to the fractionation steps verified by re-analyzing an aliquot of the recollected volatiles by GC-FID. For each fraction, a separate recollection pipette was attached at the outlet of the GC column and cooled to 4°C with ice in order to recollect all volatiles after GC-separation. Ten aliquots of 5 µl silica extract were recollected on one pipette in order to obtain sufficient material for one bioassay. All volatiles were desorbed from the SuperQ filters with 200 µl dichloromethane and applied to filter paper disks for the bioassay as described below. The recollection efficiency of the preparative GC procedure was calculated by injecting a mixture of synthetic volatile compounds with known concentration and was ≥80% except for (*Z*)-3-hexenal that was recollected with approximately 66% efficiency (data not shown).

To separate non-polar from polar compounds in the silica extract we first desorbed the volatiles collected on the silica filters with 300 µl methanol (Suprasolve, GC-grade, Merck, Darmstadt, Germany). The silica extract in methanol similarly was attractive to the wasps just as the silica extract obtained with dichloromethane (data not shown), and it contained the same major compounds as found in the dichloromethane (Fig. 5). Subsequently, 1 ml of hexane was added to 1 ml of the silica extract in methanol, thoroughly shaken, and placed in the −80°C freezer in order to separate the non-polar hexane phase from the polar methanol phase. Each phase was transferred to a separate vial by a 1 ml GC-syringe and stored in the freezer until used for the bioassays as described below.

To identify the volatile compounds, at least one 2-µl aliquot of the silica extract and the breakthrough extract were injected in a gas chromatograph (Agilent 6890 Series GC system G1530 A) coupled to a mass spectrometer that operated in the electron impact mode (Agilent 5973 Network Mass Selective Detector; transfer line 230°C, source 230°C, ionization potential 70 eV, scan range 33–280 amu) in the pulsed splitless mode onto either the non-polar or the polar column with helium at constant flow (0.9 ml/min) as carrier gas. Oven temperature and ramp were similar to that described above for the preparative GC analyses. The identities of volatiles were confirmed by comparing their mass spectra with those of the NIST 02 library

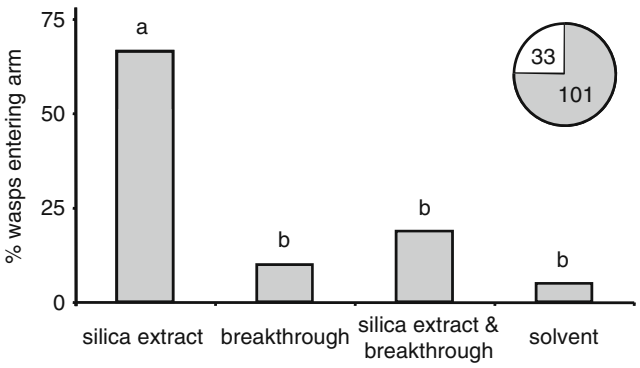

Fig. 1 Olfactometer responses of *Cotesia marginiventris* to different volatile extracts of a blend with volatile compounds emitted by *Spodoptera*-infested maize seedlings. Chemical composition of the extracts are given in Fig. 2. The pie-charts indicate the number of wasps that entered an olfactometer arm (*grey*) and the number of wasps that did not make any choice and remained in the center of the olfactometer (*white*). Different letters above the bars indicate significant differences among the various extracts (GLM: $P<0.05$)

and by comparing the retention times with those of previous analyses (D'Alessandro and Turlings 2005).

Olfactometer Bioassays

All extracts and fractions were tested for attractiveness to parasitoids in a four-arm olfactometer as described by D'Alessandro and Turlings (2005). Cleaned and humidified air entered the odor source vessels at 1.2 l/min (adjusted by a manifold with four flowmeters; Analytical Research System, Gainesville, FL, USA) via Teflon tubing and carried the volatiles through to the olfactometer compartment. Half of the air (0.6 l/min/olfactometer arm) was pulled out via a volatile collection trap that was attached to the system above the odor source vessels. An aliquot of 100 µl of each extract or 50 µl of the fractions obtained by preparative GC was placed on a filter paper (1/2 disk, 55 mm diam, Schleicher & Schuell GmbH, Dassel, Germany) that was inserted in the glass tube connecting the odor source vessels to the olfactometer arms. After letting the solvent evaporate for 2 h, wasps were released in groups of 6 into the central part of the olfactometer. Wasps that had entered an arm of the olfactometer after 30 min were counted and removed. Wasps that did not enter an arm after this time were removed from the central part of the olfactometer and considered as "no choice". Bioassays were replicated at 4 to 8 d, and for each replicate a total of four groups of 6 wasps were tested as described before

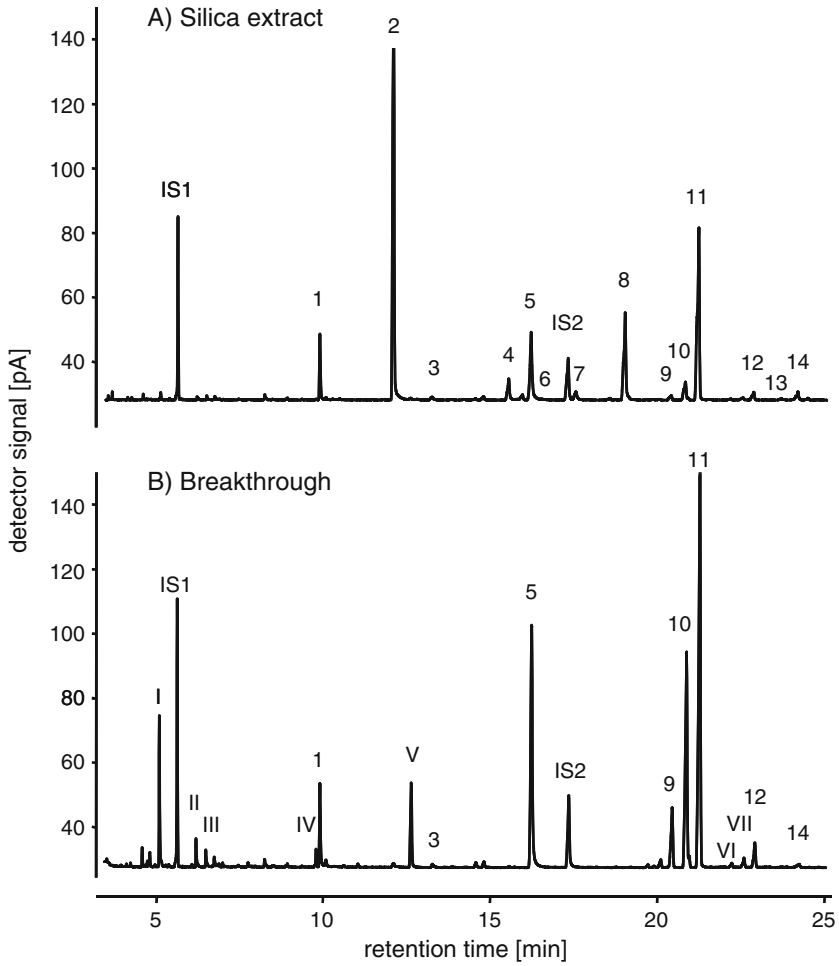

Fig. 2 GC-FID chromatograms of different extracts of a blend with volatile compounds emitted *Spodoptera*-infested maize seedlings that were obtained by passing the entire blend over a silica containing filter tube. Silica extract = volatiles retained in the silica filter. Breakthrough = volatiles that broke through the filter. The entire blend of *Spodptera*-infested maize seedlings contains the combination of both chromatograms. The compounds are: 1 = (*Z*)-3-hexenyl acetate, 2 = linalool, 3 = benzyl acetate, 4 = phenethyl acetate, 5 = indole, 6 = unknown compound, 7 = methyl anthranilate, 8 = geranyl acetate, 9 = (*E*)-β-caryophyllene, 10 = (*E*)-α-bergamotene, 11 = (*E*)-β-farnesene, 12 = β-sesquiphellandrene, 13 = (*E*)-nerolidol, 14 = (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT), I = (*Z*)-3-hexenal, II = (*E*)-2-hexenal , III = (*Z*)-3-hexen-1-ol, IV = β-myrcene, V = (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), VI = unknown sesquiterpene, VII = unknown sesquiterpene. Some compounds are not appearing well as a peak on this scaling, but their location is still indicated with a number. Compounds were identified by GC-MS analyses as indicated in Methods and Materials

(Turlings et al. 2004). All bioassays were carried out between 10 A.M. and 4 P.M.

Statistical Analyses

The functional relationship between parasitoids' behavioral responses and the different volatile extracts and fractions offered in the four-arm olfactometer was examined with a generalized linear model as described earlier by D'Alessandro and Turlings (2005). The model was fitted by maximum quasi-likelihood estimation in the software package R (R: A language and Environment for Statistical Computing, Version 1.9.1, Vienna, Austria, 2006, ISBN 3-900051-07-0, http://www.R-project.org), and its adequacy was assessed through likelihood ratio statistics and examination of residuals. Due to possible differences in the evaporation rate of individual compounds from filter papers, we included the time effect as an explanatory variable in the statistics. However, in none of the experiments was a time effect found, so only statistical values of the treatment effects (volatile extract or fraction) are indicated in the results.

Results

Fractionation over Selective Filter Tubes

In a first four-arm olfactometer experiment, we compared the attraction of females of the parasitoid *C. marginiventris* to the volatile compounds emitted by *Spodoptera*-infested maize seedlings that were trapped on a silica containing filter tube (silica extract) to the ones that broke through the filter (breakthrough), as well as to a reconstitution of the whole volatile blend (silica extract and breakthrough)

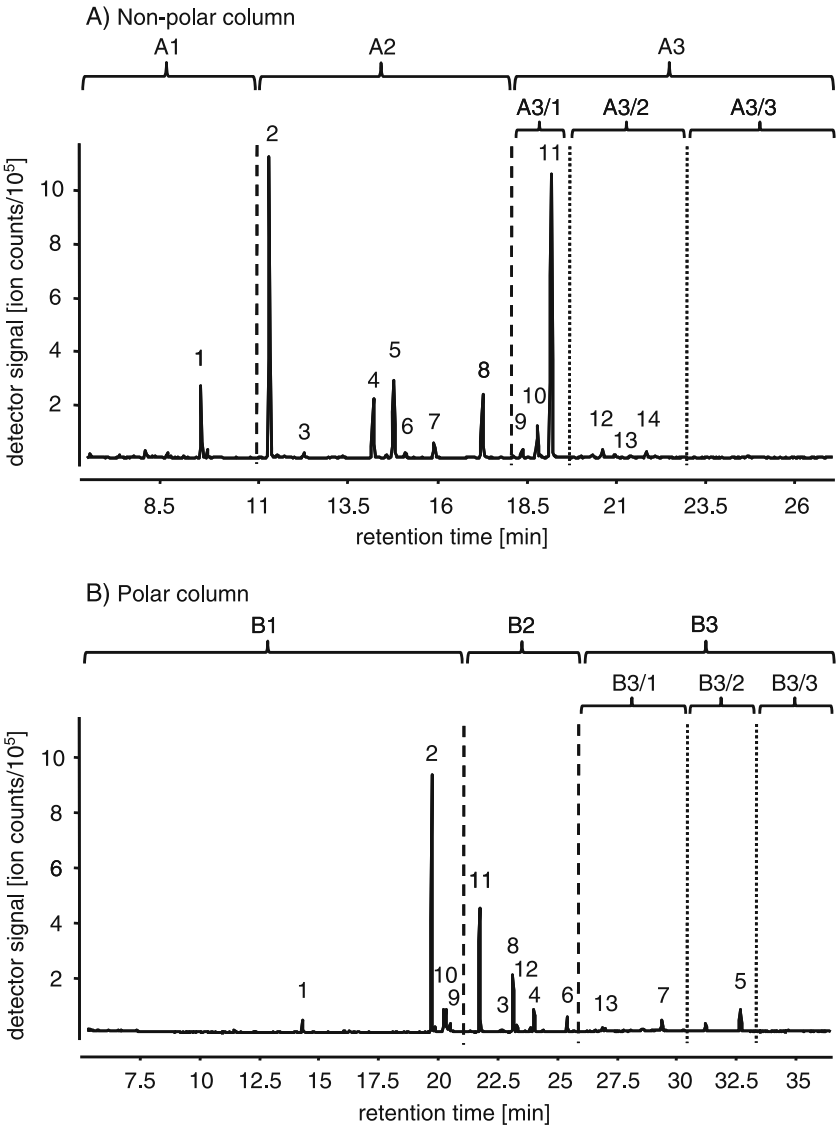

Fig. 3 GC-MS chromatograms of the highly attractive silica extract in dichloromethane indicating different fractions that were isolated by preparative GC on two different columns. The identity of the compounds is given in Fig. 2

and to the solvent only. There was a significant difference in the attractiveness of these various odor sources (GLM: $F_{3,92} = 14.19$, $P<0.001$) (Fig. 1). As in the previous study (D'Alessandro and Turlings 2005), naïve *C. marginiventris* females were extremely attracted to the silica extract, but not to the volatile blend that broke through the filter (Figs. 1 and 2). Interestingly, the silica extract was also more than 3 times as attractive as the reconstitution of the whole blend. The precise composition of each volatile extract is shown in Fig. 2. The amounts of the individual compounds found in the different extracts were not quantified in this study, but the chromatographic analyses of the extracts indicated that they were similar to the previous study (D'Alessandro and Turlings 2005).

Fractionation by Preparative GC

In order to determine the most attractive compounds in the attractive silica extract, the extract was further fractionated into three volatile extracts by preparative gas chromatography (preparative GC) on a non-polar GC-column (HP-1 MS) (Fig. 3A: A1, A2, A3) and tested for attraction in the olfactometer. Significantly more wasps entered the arm with fraction A3 than with fractions A1 and A2, but the latter were still more attractive than solvent only (Fig. 4A; GLM: $F_{3,124} = 14.19$, $P<0.001$). Subsequently, the most attractive fraction of the silica extract was again fractionated into three extracts (Fig. 3A: A3/1, A3/2, A3/3) and tested for attraction. Fraction A3/2 was not attractive, while fractions A3/1 and A3/3 were similarly attractive to the wasps (Fig. 4A; GLM: $F_{3,116} = 4.07$, $P<0.01$).

A second fractionation of the silica extract was carried out on a polar column (HP-Innowax) (Fig. 3B). The most attractive fraction resulting from a first fractionation step was fraction B3, but all fractions with HIPV were significantly more attractive than solvent only (Fig. 4B; GLM: $F_{3,124} = 7.73$, $P<0.001$). Further fractionation of the most attractive fraction B3 resulted in two fraction, B3/1 and B3/2, that were more attractive than solvent only (Fig. 4B; GLM: $F_{3,60} = 14.60$, $P<0.001$).

Fractionation with Different Solvents

In a subsequent experiment, we separated less polar from more polar compounds by the use of different solvents. Volatiles adsorbed on the silica filter were first desorbed with methanol. This methanol extract was similarly as attractive as the dichloromethane extract used in the previous experiments (data not shown), and both of these extracts contained the same major HIPV (Figs. 2 and 5). By adding a similar amount of hexane to the silica extract in methanol, we obtained a hexane phase that contained non-polar compounds and a methanol phase with polar compounds (Fig. 5). In olfactometer bioassays, we found that

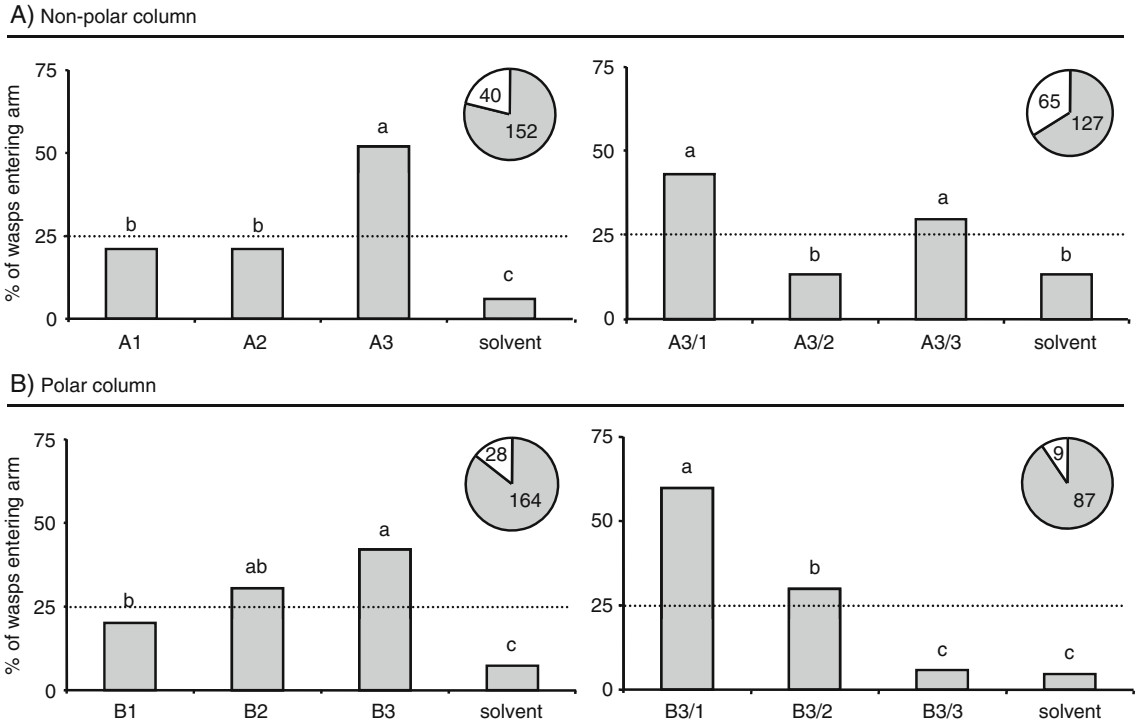

Fig. 4 Olfactometer responses of *Cotesia marginiventris* to different fractions of the silica extract that were isolated by preparative GC as indicated in Fig. 3. Explanations to the pie-charts and to the statistics are given in Fig. 1

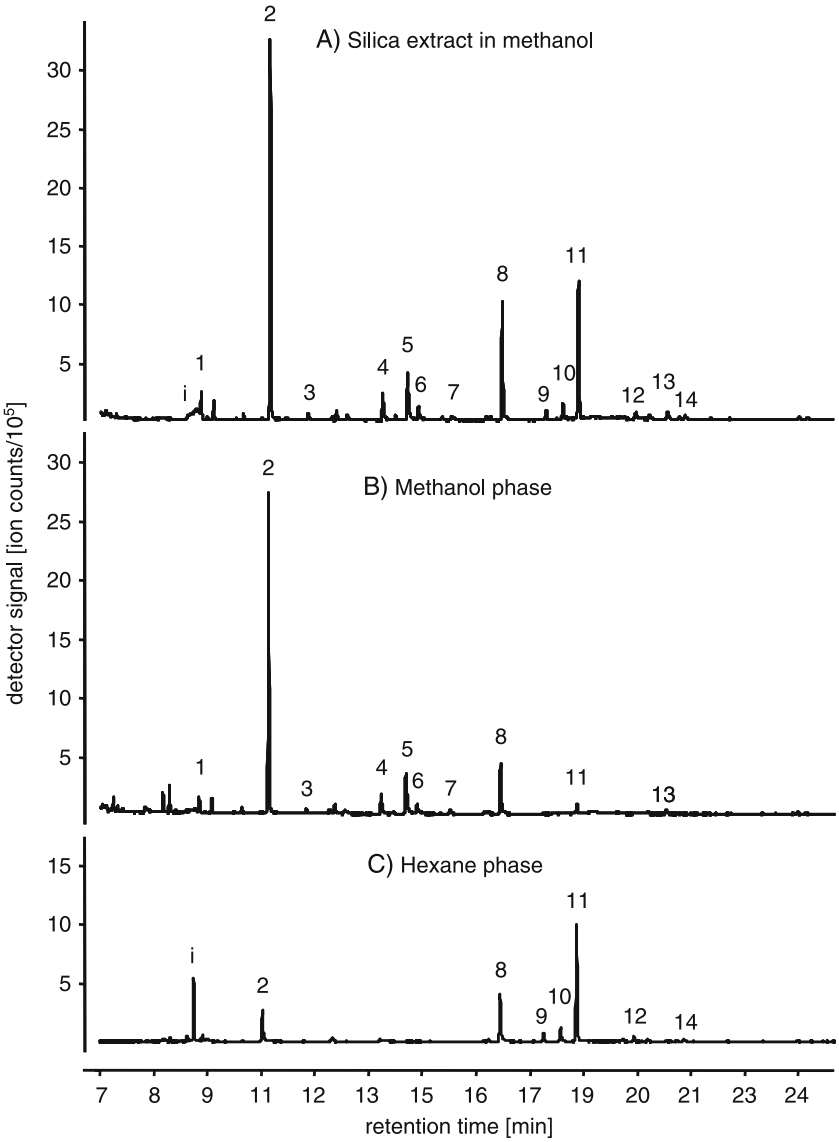

Fig. 5 GC-MS chromatograms of the highly attractive silica extract in methanol and different fractions that were isolated by using solvents with different polarities. The identity of the compounds is given in Fig. 2

the polar methanol phase was significantly more attractive than the less polar hexane phase, but the latter was more attractive than the solvents only (Fig. 6A, GLM: $F_{3,92}$ = 65.60, P<0.001). Comparing the methanol phase to the whole silica extract in methanol showed that the latter was significantly more attractive than the extract without the less polar compounds, indicating that some attractive compounds are soluble in hexane and were either missing or strongly reduced in the methanol phase (Fig. 6B, GLM: $F_{1,46}$ = 6.68, P<0.05).

Discussion

In this study, we used a combination of fractionation methods and olfactometer bioassays to characterize the most attractive compounds of the volatile blend emitted by *Spodoptera*-infested maize seedlings that are used by the solitary endoparasitoid *C. marginiventris* as host-location cue. Similarly, as in a previous study (D'Alessandro and Turlings 2005), the extract that was obtained by passing the entire HIPV-blend over a silica filter tube and contained rather polar compounds and was highly attractive to naive *C. marginiventris* females, whereas the breakthrough extract was not attractive at all to this wasp even though it also contained most measurable HIPV (Figs. 1 and 2). Further separation of less polar from more polar compounds in the silica extract proved that the wasp preferred polar over non-polar compounds (Figs. 5 and 6). However, the detectable polar compounds that were present in the attractive silica extract but not in the non-attractive breakthrough extract (compounds 2, 4, 6, 7, 8, and 13) could not explain the high attractiveness of the silica extract. First, a combination of synthetic versions of

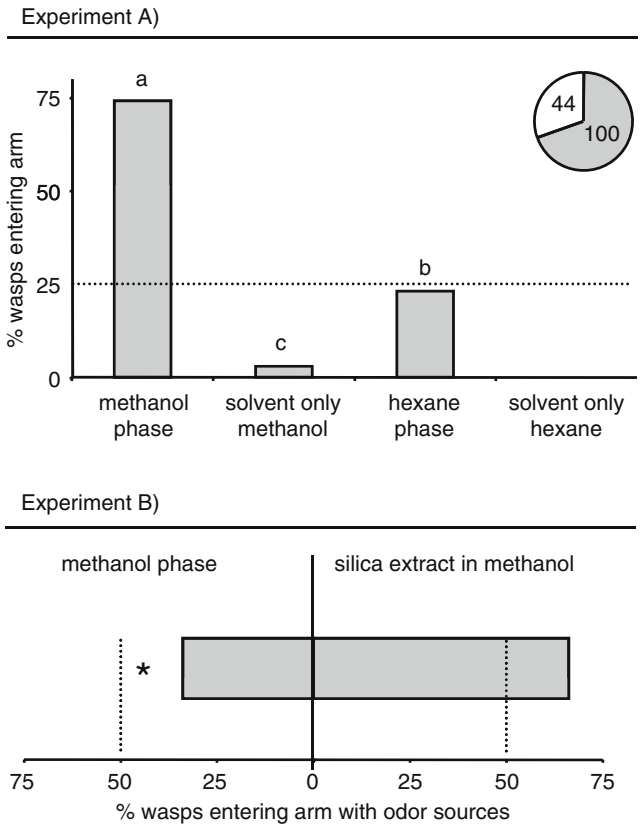

Fig. 6 Olfactometer responses of *Cotesia marginiventris* to different volatile fractions that were isolated by using solvents with different polarities as indicated in Fig. 5. A) Comparison of the polar and the non-polar fraction as well as the solvents as control. Explanations to the pie-charts and to the statistics are given in Fig. 1. B) Comparison of the polar fraction to the entire silica extract in methanol

compounds 2, 4, 7, 8, and 13 was not attractive to the wasps (data not shown). Second, further fractionation of the silica extract by preparative GC did not result in a clear allocation of these compounds to attractive and non-attractive fractions. For instance, although compound 13 was present in the most attractive fraction in some bioassays (fractions A3, B3, and in B3/1), in others it was present in the least attractive fraction (fraction A3/2) (Figs. 3 and 4). Similarly, compound 7 was present in the highly attractive fraction (B3/1), but fraction A2 was significantly less attractive than fraction A3 although it also contained this compound as well as most of other compounds that were present in the highly attractive silica extract. Finally, the unknown compound 6 was present in fractions with medium attractiveness. Overall, our experiments suggest that the compounds detected in the chemical analyses, are not likely to be the most attractive ones, nor the only ones that are needed by *C. marginiventris* as host location cues.

Another interesting observation was that the silica extract, which contained only a fraction of the whole blend, was by far more attractive than the entire blend of herbivore-induced maize volatiles (Figs. 1 and 2). This suggests that certain compounds within a blend of HIPV have either repellent effects on the attraction of *C. marginiventris* or are masking the attractiveness of other attractive compounds. Similar findings have been found in earlier studies and with other insect species. For instance, naïve females of the parasitoid *Microplites rufiventris* clearly preferred volatile blends that did not contain indole, a major HIPV of maize, over blends that contained indole at normal concentration, and this difference was due to a masking effect of the volatile compound indole itself (D'Alessandro et al. 2006). Another common plant compound that recently has been claimed to interfere with the attraction of certain parasitic wasps is isoprene (Loivamaki et al. 2008). By contrast, other studies have shown synergistic effects of certain volatile compounds. For instance, neither nonanal nor geranylacetone alone was attractive to the parasitoid females of *Apanteles carpatus*, a parsitoid of the cloth moth *Tineae pennionella*, but a one-to-one blend of both compounds was as attractive as an extract of all volatile compounds from moth-infested beaver pelt (Takács et al. 1997). Similarly, the egg parasitoid *Chrysonotomyia ruforum* of the herbivorous sawfly *Diprion pini* did not respond to the sesquiterpene (*E*)-β-farnesene, an oviposition-induced pine twig volatile, if offered to the females as an individual compound at different doses (Mumm and Hilker 2005). However, the parasitoid was significantly attracted to this compound when tested together with the odor of pine twigs without eggs. These studies illustrate, that optimal attraction requires the presence of a specific combination of compounds. Unfortunately, so far no general pattern could have been recognized that would help to determine which compounds reduce or enhance the attractiveness of odor sources to a foraging insect (reviewed by Schröder and Hilker 2008).

One reason for the lack of a clear understanding of synergistic and antagonistic effects of individual compounds within complex blends might be the fact that insects respond to volatile compounds in a dose-dependent manner (De Boer and Dicke 2004; Ngumbi et al. 2005; Yan and Wang 2006). Such effects also have been reported in earlier studies with the parasitoid *C. marginiventris* (Fritzsche Hoballah et al. 2002; Turlings et al. 2004). The surprising finding in this study was the strong attraction of *C. marginiventris* to some fractions that contained volatile compounds in extremely low quantities (e.g., A3/3). In fact, the concentrations of these compounds were below the detection limit of the GC analyses, i.e., in the pico-gram range or lower, which has made it impossible to identify the compounds thus far. Previous electrophysiological analyses with *C. marginiventris* showed that some HIPV elicited strong electroantennographic responses in the antennae of the insects at concentrations

below the detection level of the GC analyses (Gouinguené et al. 2005). These observations strongly suggest that minor, yet unknown compounds, play a crucial role in the attraction of *C. marginiventris* to *Spodoptera*-induced maize volatiles. In fact, the olfactory sensitivity of insects is astonishing. For instance, by using the moth *Spodoptera littoralis,* it was nicely demonstrated that around five molecules of the sex pheromone and around ten molecules of a specific plant odor hitting the antenna during one second were sufficient to trigger a heartbeat frequency change (Angioy et al. 2003). It is likely that compounds emitted in such low quantities are likely to escape chemical analysis.

Another complicating factor in identifying minor compounds that are highly attractive to parasitoids is the specific chemical structure of some volatiles. The chemical diversity of volatiles compounds emitted by plants is enormous, ranging from alkanes, alkenes, alcohols, ketones, aldehydes, ethers, and esters to carboxylic acids and others (Niinemets et al. 2004). It is likely that a single volatile sampling and analyses method cannot provide the entire picture of the qualitative and quantitative composition of an herbivore-induced volatile blend. In this study, it is possible that key compounds did not chromatograph well and either did not elute or were eluted throughout the analyses by "bleeding" off the column. This latter possibility could also explain the attractiveness of multiple fractions that might all have contained elusive compound(s). Thus, alternative approaches to conventional volatile collection methods and GC analysis are needed to unravel the identity of highly attractive minor compounds and for a better understanding of the attractiveness HIPV-blends in general. Recent studies that have applied novel methods, such as PTR-MS, have revealed the presence of methanol in the volatile blend of a number of herbivore-infested plants, a compound, that has not been detected with conventional trapping and GC analysis methodologies (Penuelas et al. 2005; von Dahl et al. 2006). Other promising approaches might benefit from novel statistical tools (van Dam and Poppy 2008; Pareja et al. 2009) or also might consider that some plant volatiles are suppressed rather than induced upon insect infestation (Gaquerel et al. 2009). In conclusion, when new methods are used to analyze HIPV blends in a more comprehensive manner, the eventual identification of key attractants or repellents for parasitoids and predators may provide potential to improve biological control of insect pests. The transformation of maize plants with a gene responsible for the biosynthesis of a (*E*)-ß-caryophyllene, a key volatile attractant for entomopathogenic nematodes of the Western corn rootworm, is a recent example of how the HIPV-blend can be modified to successfully control this ferocious root pest of maize plants (Degenhardt et al. 2009).

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et al. 2005). In contrast, HIPVs are emitted as a delayed response to herbivore feeding damage. HIPVs in cotton (*Gossypium hirsutum* L) and other plants include *cis*-3-hexenyl acetate, *cis*-3-hexenyl butyrate, indole, and various terpenoids such as (*E,E*)-α-farnesene, (*E*)-β-farnesene, (*E*)-β-ocimene, and linalool (Dicke 1994; Loughrin et al. 1994; McCall et al. 1994; Cortesero et al. 1997).

Although, the emission of volatiles is assumed to represent a generalized response to herbivore damage, the blends of volatile compounds released from herbivore damaged plants differ qualitatively and quantitatively depending on the plant species and variety (Dicke et al. 1990; Loughrin et al. 1994; Hoballah et al. 2002), the herbivore species (De Moraes et al. 1998; Loughrin et al. 1994; McCall et al. 1994), and the stage of the herbivore (Takabayashi et al. 1991; Du et al. 1996). For instance, corn (*Zea mays* L.) plants infested by beet armyworm *Spodoptera exigua* (Hübner) caterpillars emit linalool, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, (*trans*)-α-bergamotene, and (*E*)-β-farnesene as major compounds, all of which have not been detected in the headspace of soybean (*Glycine max* L.) plants infested by the same species (Turlings et al. 1993). In cotton, feeding by corn earworm *Helicoverpa zea* (Boddie) or *S. exigua* caterpillars induces the production of distinctive volatile blends that are qualitatively and quantitatively different (Loughrin et al. 1994; McCall et al. 1994). McCall et al. (1994) reported that cotton plants damaged by *H. zea* emit several compounds including (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, (*Z*)-3-hexenyl butyrate, (*E*)-2-hexenyl butyrate, (*Z*)-3-hexenyl-2-methylbutyrate, (*E*)-2-hexenyl-2-methylbutyrate, and indole. Loughrin et al. (1994) conducted a similar study with cotton plants damaged by *S. exigua* and reported several compounds including some of the above, and many which were not reported by McCall et al. (1994) such as (*Z*)-jasmone, (*E*)-β-farnesene, and (*E,E*)-α-farnesene. Such differences in the composition of volatiles induced by different herbivores may convey herbivore-specific information to parasitoids, and thus shape their foraging strategies (Dicke and Sabelis 1988; Turlings et al. 1990, 1995; McCall et al. 1993). In particular, the volatile blend signature produced by plants in response to different herbivores may be used by specialist parasitoids as signals for host specificity (Du et al. 1996; De Moraes et al. 1998). For instance, the specialist parasitoid *Cardiochiles nigriceps* Viereck can exploit differences in volatile blends produced by cotton or corn plants in response to different herbivores, thus distinguishing infestation by its host *H. virescens* from that of the closely related *H. zea* (De Moraes et al. 1998).

The question of whether specialist and generalist parasitoids show differential responses to different suites of host-related volatiles has been a major focus of evolutionary ecology in recent years (Vet et al. 1993; Geervliet et al. 1996; Bernays 2001; Chen and Fadamiro 2007; Stilmant et al. 2008). It has been predicted that specialist parasitoids that utilize fewer numbers of hosts are likely to possess a more highly sensitive (high olfactory sensitivity to host-related chemical cues) and narrowly-tuned (selective) host detection olfactory system than generalist parasitoids (Vet and Dicke 1992; Cortesero et al. 1997; Smid et al. 2002; Chen and Fadamiro 2007). However, few studies have compared olfactory response and sensitivity to host-related volatiles in specialist and generalist parasitoids, and they have produced contrasting results (Elzen et al. 1987; Vet et al. 1993; Geervliet et al. 1996; Chen and Fadamiro 2007). On the one hand, some studies have reported relatively greater response for specialists compared to generalists (Elzen et al. 1987; Vet et al. 1993). Additionally, Geervliet et al. (1996) recorded no differences in behavioral responses of the specialist, *Cotesia rubecula* Marshall and the generalist, *Cotesia marginiventris* (Cresson) to host-related volatiles, and both species were unable to distinguish between plant volatiles induced by their hosts vs. those induced by non-host species. Similarly, Smid et al. (2002) reported no differences in the receptive range of the specialist, *C. rubecula* and the generalist, *Cotesia glomerata* L. to a wide range of host-related odor compounds. Such discrepancies suggest that diverse species of specialist and generalist parasitoids may respond differently to different types of host-related volatiles. Furthermore, even within a broad category of specialist or generalist parasitoids, differences may exist among species based on the degree of specialization (De Moraes et al. 1998; Tamo et al. 2006).

In this study, we tested the above prediction by using a tritrophic model system consisting of cotton (plant), *H. zea* and *S. exigua* (herbivores), and two parasitoids (Hymenoptera: Braconidae) with different degrees of host specificity, *Microplitis croceipes* (Cresson) and *C. marginiventris*. *Microplitis croceipes* is a relatively specialist parasitoid specific to the caterpillars of *H. zea* and *H. virescens*, while *C. marginiventris* is a generalist parasitoid of caterpillars of a wide range of lepidopteran species, including *S. exigua*, *H. zea*, *H. virescens* (Jalali et al. 1987; Turlings et al. 1990; Lewis et al. 1991; Röse et al. 1998). Both parasitoids were selected as experimental models for this comparative study because they have served as models in previous studies of parasitoid olfaction, and because several aspects of their responses to host-related volatiles have been characterized (e.g., Dmoch et al. 1985; Li et al. 1992; Cortesero et al. 1997; Röse et al. 1998; Park et al. 2002; Gouinguené et al. 2005). We used the coupled gas chromatography electroantennogram detection (GC-EAD) technique to test for similarities and differences in antennal responses of both parasitoid species to headspace volatiles of cotton plants infested with *H. virescens* (a host species for both parasitoids) vs. *S. exigua* (a host species for *C. marginiventris*

but not for *M. croceipes*). Based on the results of a recent study in which we recorded differences in the electroantennogram (EAG) responses of both parasitoids to various synthetic host-related volatile compounds (Chen and Fadamiro 2007), we hypothesized that *M. croceipes* would show relatively greater GC-EAD responses than *C. marginiventris* (generalist) to the HIPV components of cotton headspaces, whereas the GLV components, which are emitted passively by plants and as a generalized response to herbivore damage, would elicit relatively greater GC-EAD activity in the generalist.

Methods and Materials

Plants

Cotton (*G. hirsutum*, var. max 9) plants were grown in individual pots (9 cm high, 11 cm diam) in a greenhouse (Auburn University Plant Science Greenhouse Facility) at 25°C±10, 15:9 h (L/D) photoperiod and 50±10% relative humidity. Seeds were planted in a top soil/vermiculate/peat moss mixture. Plants used for headspace volatile collections were 4–6 wk-old.

Caterpillars (Parasitoid Hosts)

Two lepidopteran species, *H. virescens* and *S. exigua* were used as parasitoid hosts. Both species are distributed throughout the United States and are important pests of agricultural crops including corn, and cotton. Eggs purchased from Benzon Research (Carlisle, PA, USA) were used to start laboratory colonies. Caterpillars of both species were reared on a laboratory-prepared pinto bean diet (Shorey and Hale 1965) at 25±1°C, 75±5% relative humidity and 14:10-h (L/D) photoperiod.

Parasitoids

The parent cultures of *M. croceipes* and *C. marginiventris* were provided by the USDA-ARS, Insect Biology and Population Management Research Laboratory (Tifton, Georgia) and the University of Georgia, Tifton campus (contact: John Ruberson), respectively. *Microplitis croceipes* was reared on caterpillars of *H. virescens*, its preferred host (Stadelbacher et al. 1984; King et al. 1985), whereas *C. marginiventris* was reared on caterpillars of its main host *S. exigua* (Jalali et al. 1987). The rearing procedures were similar to those of Lewis and Burton (1970), and the rearing conditions were the same as described above for the caterpillar hosts. For each species, newly emerged adults were collected prior to mating, sexed, and placed in groups of 2 individuals of opposite sex (mated individuals) in a 6-cm diam plastic Petri dish supplied with water and sugar sources. Water was provided by filling a 0.5 ml microcentrifuge tube with distilled water and threading a cotton string through a hole in the cap of the tube. About 4–6 drops (2 µl per drop) of 10% sugar solution were smeared on the inside of the Petri dish cover with a cotton-tipped applicator. Female parasitoids (aged 3–5 d-old) of both species were used.

Collection and GC Analysis of Headspace Volatiles

The methodology and protocols used for volatile collection were similar to those reported by Gouinguené et al. (2005), but with some modifications. Headspace volatiles were collected both from caterpillar damaged and undamaged cotton plants. To induce the production of HIPVs from plants, 30 second instars of *H. virescens* or *S. exigua* were allowed to feed on a potted cotton plant for 12 h prior to volatile collection. The pot with the potting soil was wrapped with aluminum foil to minimize evaporation of water and volatiles. The plant (with the feeding caterpillars) was then placed in a volatile collection chamber (Analytical Research Systems, Inc., Gainesville, FL, USA) consisting of a 5 l glass jar. A purified (activated charcoal) air stream of 500 ml/min was passed through the jar at room temperature for 24 h. The results of a pilot test that compared headspace volatile collection for 24 h vs. 12 h showed no noticeable differences in the number or relative proportion of the peaks, however the 24 h duration was selected because it produced consistent profiles in which all key peaks were detected in relatively higher amounts. Headspace volatiles were trapped with a trap containing 50 mg of Super-Q (Alltech Associates, Deerfield, IL, USA) and eluted with 200 µl of methylene chloride. The resulting extracts (200 µl) were stored in a freezer (at −20°C) until use. Another container with potting soil without plant was used to check for miscellaneous impurities and background noise. The collection system was checked and controlled for breakthrough of the trap during sampling. One µl of each headspace volatile extract was injected into a Shimadzu GC-17A equipped with a flame ionization detector (FID). The dimension of capillary column used was as follows: Rtx-1MS, 0.25 mm I.D., 0.25 µm film thickness (Restek, Bellefonte, PA, USA). Helium was used as carrier gas at a flow rate of 1 ml/min. The GC oven was programmed as follows: inject at 40°C, hold at 40°C for 2 min, and then increase by 5°C/min to 200°C for a total of 40 min. The temperature of both injector and detector was set at 200°C.

GC-EAD Recordings

The extracts were subjected to coupled gas chromatography-electroantennogram detection (GC-EAD) analyses with

females of both parasitoids to detect biologically active peaks (components). GC-EAD analyses were conducted with samples of headspace volatiles from cotton plants infested with *H. virescens* or *S. exigua* caterpillars and detected with antennae of *M. croceipes* or *C. marginiventris* females (total of 4 combinations or treatments). The GC-EAD techniques used were similar to those described by Smid et al. (2002). Briefly, the system was based on the above Shimadzu GC-17A equipped with a FID and coupled to an EAG detector. The dimension of the GC capillary column was the same as described above. The column effluent was mixed with 30 ml/min make-up helium and split at a ratio of 1:2 (v/v), with one part going to the FID and the other through a heated (220°C) transfer line (Syntech®, Hilversum, the Netherlands) into a charcoal filtered, humidified airstream (1000 ml/min) directed at the antenna preparation (EAG detector). The GC oven was programmed as above. The antenna preparation and EAG techniques were the same as previously described (Chen and Fadamiro 2007). Glass capillaries (1.1 mm I.D.) filled with Ringer solution were used as electrodes. Parasitoids were first anaesthetized by chilling, and the head was isolated. The reference electrode was connected to the neck of the isolated head, while the recording electrode was connected to the antennal tip (with the last segment of antenna cut off). Chlorinated silver-silver chloride junctions were used to maintain electrical contact between the electrodes and input of a 1× preamplifier (Syntech®). The analog signal was detected through a probe (INR-II, Syntech®), captured and processed with a data acquisition controller (IDAC-4, Syntech®), and later analyzed with software (GcEad 32, Syntech®) on a personal computer. A 3-µl aliquot of each sample was injected for a GC-EAD run. Five successful GC-EAD recordings were obtained for each treatment. GC-EAD traces were overlaid on the computer monitor and inspected for significant and consistent qualitative and quantitative differences among treatments.

GC-MS Analyses

The GC-EAD active peaks in each treatment were identified by gas chromatography-mass spectrometry (GC-MS) using an Agilent 7890A GC coupled to a 5975C Mass Selective Detector, with an HP-5 ms capillary column (30 m×0.25 mm I.D., 0.25 µm film thickness). One µl of each headspace extract was injected into the GC in splitless mode and under the GC conditions described above for GC-EAD. The chromatographic profiles were similar to those obtained from GC-EAD recordings, thus making it possible to match the peaks. Mass spectra were obtained by using electron impact (EI, 70 eV). Identification of EAD-active peaks was done by using NIST 98 library (National Institute of Standards and Technology, Gaithersburg, Maryland) and by comparing with published GC profiles of cotton head space volatiles (Thompson et al. 1971; Loughrin et al. 1994; McCall et al. 1994). The structures of the identified compounds were confirmed by using commercially available synthetic standards with purity >97% (as indicated on the labels) obtained from Sigma® Chemical Co. (St. Louis, MO, USA). Significant differences in the amounts of each volatile component emitted by *H. virescens* damaged vs. *S. exigua* damaged cotton plants were established by using the Student's *t*-test ($P<0.05$, JMP® 7.0.1, SAS Institute 2007).

GC-EAD Analyses with Synthetic Blend

In order to confirm the observed differences in the GC-EAD responses of both parasitoids to the headspace extracts, a synthetic blend that mimicked the headspace of caterpillar-infested cotton plants was prepared. This blend was formulated to mimic closely the active components of the headspace of cotton plants infested with *H. virescens*, although the same compounds were detected also in the headspace of cotton plants infested with *S. exigua.* It consisted of 13 synthetic volatile compounds that were identified as key biologically active components in the headspace volatiles of cotton plants infested with *H. virescens*, and blended at an approximate ratio in which they were detected in the headspace. The compounds were purchased from the above source with purity >97% and included *cis*-3-hexenal, *trans*-2-hexenal, *cis*-3-hexen-1-ol, *cis*-3-hexenyl acetate, *trans* -2-hexenyl acetate, linalool, *cis*-3-hexenyl butyrate, *trans*-2-hexenyl butyrate, indole, *cis*-jasmone, α-farnesene, α-humulene, and *trans*-nerolidol, blended in the ratio of 4.8, 7.8, 1.9, 19.8, 12.2, 2.2, 13.3, 11.1, 7.2, 0.4, 4.6, 4.3, and 10.2, respectively. Each compound was diluted in hexane and blended at the above ratio to obtain a 100 µg/µl solution. A 3-µl aliquot of the blend (100 µg/µl) was injected for a GC-EAD run. Five successful GC-EAD recordings were obtained for each parasitoid species as described above.

Quantification of GC-EAD Responses

GC-EAD responses of both parasitoids to different volatile components were quantified by using a measurement marker tool available with the GC-EAD software (GcEad 32). This tool enabled the quantification of EAD peaks in microvolts (µV). Significant differences in GC-EAD responses of both parasitoid wasps to each volatile component were then established by using the Student's *t*-test ($P<0.05$: JMP® 7.0.1, SAS Institute 2007).

Results

GC and GC-MS Analysis of Headspace Volatiles

The GC profiles of the extracts of headspace volatiles from cotton plants infested with *H. virescens* or *S. exigua* vs. uninfested (undamaged) plants are shown in Fig. 1. A total of 30 peaks (volatile components) were detected in the headspace of plants infested with *H. virescens* or *S. exigua* (Fig.1a, b). Identical compounds were detected in both extracts, meaning that no qualitative differences were recorded. However, noticeable *quantitative* differences were recorded. In particular, 18 peaks were significantly elevated in the headspace of plants infested with *H. virescens* compared to those infested with *S. exigua* (Table 1). These elevated peaks, as identified by GC-MS, included *cis*-3-hexenal, *cis*-3-hexen-1-ol, α-pinene, β-myrcene, *cis*-3-hexenyl butyrate, *cis*-3-hexenyl-2-methyl butyrate, *cis*-jasmone, α-farnesene, *trans*-nerolidol, and several other HIPV components. No peaks were elevated obviously in the headspace of plants infested with *S. exigua*, relative to those infested with *H. virescens*. Most of the above peaks were not detected or were detected in insignificant amounts in the headspace of undamaged plants (Fig. 1c). Only five peaks (components) were slightly detectable in undamaged plants and were identified by GC-MS as α-pinene, *trans*-2-hexenyl butyrate, linalool, *n*-decanal, and caryophyllene. However, all five components were detected in much greater amounts in the headspace of caterpillar-infested plants.

GC-EAD Responses

Similarities were recorded in the GC-EAD responses of *M. croceipes* and *C. marginiventris* females to volatiles from cotton infested with the two caterpillar species. Sixteen components of the headspace of caterpillar-infested plants elicited consistent GC-EAD responses in both parasitoid species (Figs. 2 and 3). As identified by GC-MS, these volatiles included several GLVs (*cis*-3-hexenal, *trans*-2-hexenal, *cis*-3-hexen-1-ol, and *trans*-2-hexen-1-ol) and HIPVs [(*E*)-4,8-dimethyl-1,3,7-nonatriene, *cis*-3-hexenyl butyrate, *trans*-2-hexenyl butyrate, *n*-decanal, *cis*-3-hexenyl-2-methyl butyrate, *trans*-2-hexenyl-2-methyl butyrate, indole, isobutyl tiglate, (*E*)-2-hexenyl tiglate, *cis*-jasmone, caryophyllene, α-*trans* bergamotene, α-farnesene, α-humulene, β-farnesene, β-hemachalene, and *trans*-nerolidol]. More importantly, key differences were recorded in

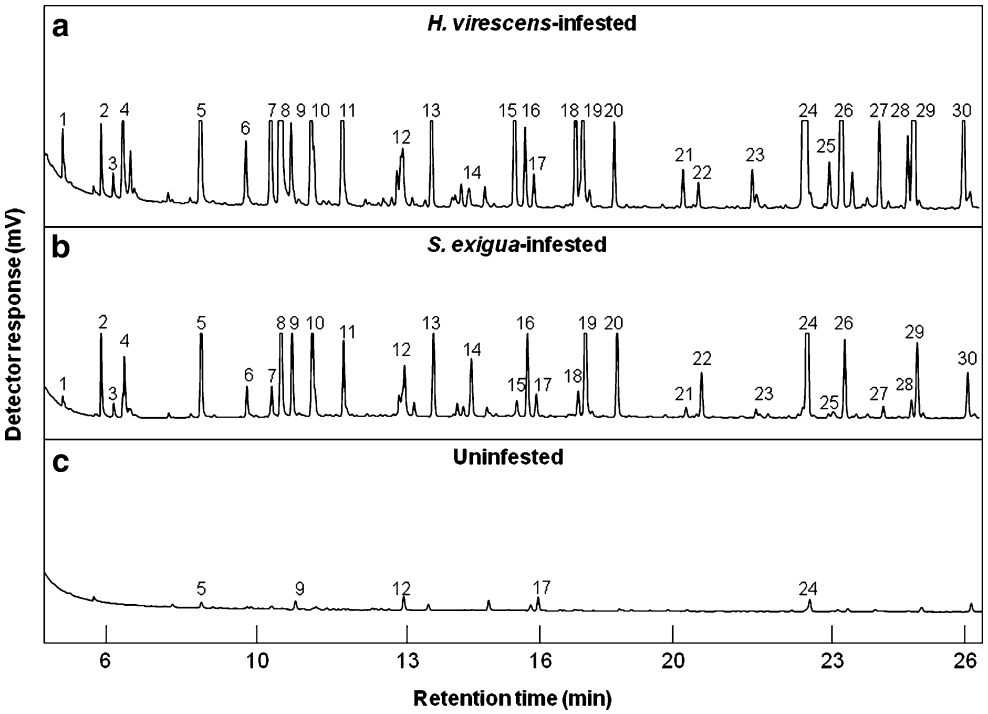

Fig. 1 Chromatographic profiles of headspace volatiles collected from cotton plants infested with *Heliothis virescens* (**a**) or *Spodoptera exigua* (**b**) caterpillars, vs. undamaged control plants (**c**). Identified compounds: (1) *cis*-3-hexenal; (2) *trans*-2-hexenal; (3) *cis*-3-hexen-1-ol; (4) *trans*-2-hexen-1-ol; (5) α-pinene; (6) β-pinene; (7) myrcene; (8) *cis*-3-hexenyl acetate; (9) *trans*-2-hexenyl acetate; (10) limonene; (11) β-ocimene; (12) linalool; (13) unknown; (14) (*E*)-4,8-dimethyl-1,3,7-nonatriene; (15) *cis*-3-hexenyl butyrate; (16) *trans*-2-hexenyl butyrate ; (17) *n*-decanal; (18) *cis*-3-hexenyl-2-methyl butyrate; (19) *trans*-2-hexenyl-2-methyl butyrate; (20) indole; (21) isobutyl tiglate; (22) (*E*)-2-hexenyl tiglate; (23) *cis*-jasmone; (24) caryophyllene; (25) α-*trans* bergamotene; (26) α-farnesene; (27) α-humulene; (28) β-farnesene; (29) β-hemachalene; (30) *trans*-nerolidol

Table 1 Composition of volatiles collected from cotton plants infested for 24 h with *Heliothis virescens* or *Spodoptera exigua* caterpillars and undamaged control plants

ID	Compound[a]	*H. virescens*-infested		*S. exigua*-infested		Uninfested	
		Amount (ng±SE)[b]	Relative %	Amount (ng±SE)[b]	Relative %	Amount (ng±SE)[b]	Relative %
1	*cis*-3-hexenal	39,350±3212[a]	1.9	1,408±238[b]	0.09	0	0
2	*trans*-2-hexenal	63,420±1106	3.0	72,438±2520	5.0	0	0
3	*cis*-3-hexen-1-ol	15,740±670[a]	0.8	8,200±720[b]	0.5	0	0
4	*trans*-2-hexen-1-ol	69,402±2230	3.3	67,120±1340	4.7	0	0
5	α-pinene	98,310±3110[a]	4.5	83,120±2620[b]	5.8	100±25	18.5
6	β-pinene	58,239 ±1939[a]	2.8	42,300±1940[b]	2.9	0	0
7	myrcene	120,259±5920[a]	5.8	15,465±853[b]	1.1	0	0
8	*cis*-3-hexenyl acetate	161,470±2350	7.7	120,475±4860	8.4	0	0
9	*trans*-2-hexenyl acetate	99,214±1074	4.8	111,345±3740	7.8	0	0
10	limonene	110,259±983[a]	5.3	84,330±750[b]	5.9	0	0
11	β-ocimene	120,257±1506[a]	5.8	89,354±2015[b]	6.2	0	0
12	linalool	18,343±939	0.9	18,468±542	1.3	150±38	27.7
13	unknown	59,320±1812	2.8	58,458±2040	4.1	0	0
14	4,8-dimethyl-1,3,7-nonatriene	21,320±1003	1.0	78,800±1296	5.5	0	0
15	*cis*-3-hexenyl butyrate	108,345±1690[a]	5.2	36,900±1165[b]	2.5	0	0
16	*trans*-2-hexenyl butyrate	90,210±4500	4.3	91,356±4300	6.4	135±60	25.0
17	*n*-decanal	5,300±412	0.3	4,800±109	0.3	75±18	13.8
18	*cis*-3-hexenyl-2-methyl butyrate	135,100±3600[a]	6.5	2,800±198[b]	0.2	0	0
19	*trans*-2-hexenyl-2-methyl butyrate	128,950±5300	6.2	115,220±5200	8.0	0	0
20	indole	58,430±1250[a]	2.8	43,200±2700[b]	3.0	0	0
21	isobutyl tiglate	15,900±840[a]	0.8	2,300±350[b]	0.2	0	0
22	2-hexenyl tiglate	6,500±152	0.3	14,999±1650	1.0	0	0
23	*cis*-jasmone	3,200±636[a]	0.2	900±330[b]	0.1	0	0
24	caryophyllene	170,500±6835	8.2	154,230±5300	10.7	80±40	14.8
25	α-*trans* bergamotene	16,378±910[a]	0.8	468±130[b]	0.03	0	0
26	α-farnesene	37,745±2470[a]	1.8	23,300±3564[b]	1.6	0	0
27	α-humulene	35,200±1119[a]	1.7	2,300±745[b]	0.2	0	0
28	β-farnesene	48,239±636a	2.3	1,305±248[b]	0.09	0	0
29	β-hemachalene	94,600±3830[a]	4.5	65,780±3200[b]	4.6	0	0
30	*trans*-nerolidol	83,170±868[a]	4.0	23,450±1950[b]	1.6	0	0

[a] In order of elution during gas chromatography

[b] Values (amount emitted) are mean±SE of five replicate extractions

Means across the same row for the same headspace extract followed by different letters are significantly different ($P<0.05$, *t*-test).

the response patterns of both parasitoids to the different components of the headspace extracts. Quantitatively, *C. marginiventris* (generalist) showed significantly greater GC-EAD responses to the GLV (e.g., *cis*-3-hexenal, *trans*-2-hexenal and *cis*-3-hexen-1-ol) components of the two extracts, compared to *M. croceipes* (specialist) (Table 2, Figs. 2, 3). In contrast, several HIPV components of both extracts (e.g., *cis*-3-hexenyl acetate, linalool, *cis*-3-hexenyl butyrate and *trans*-2-hexenyl butyrate) elicited significantly greater responses in *M. croceipes*, compared to *C. marginiventris*. In addition, α-humulene also elicited greater response in *M. croceipes* than in *C. marginiventris*, but this was significant only for *H. virescens*-infested headspace extract. *Microplitis croceipes* showed relatively greater GC-EAD responses than *C. marginiventris* to indole and *cis*-jasmone, but these differences were significant only for *S. exigua*-infested extract. Note that responses of *C. marginiventris* to some of the HIPV components were very low and barely detectable (Figs. 2, 3). In general, the GC-EAD responses of both parasitoid species to the synthetic blend mimicked their responses to the headspace volatiles of caterpillar-infested plants (Table 2, Fig. 4). A confirmatory test in which the synthetic blend was tested at a reduced amount (i.e., 1 µl of a 0.1 µg/µl solution of the blend was injected for a GC-EAD run) produced results similar to those shown in Fig. 4, suggesting that the amounts tested in the

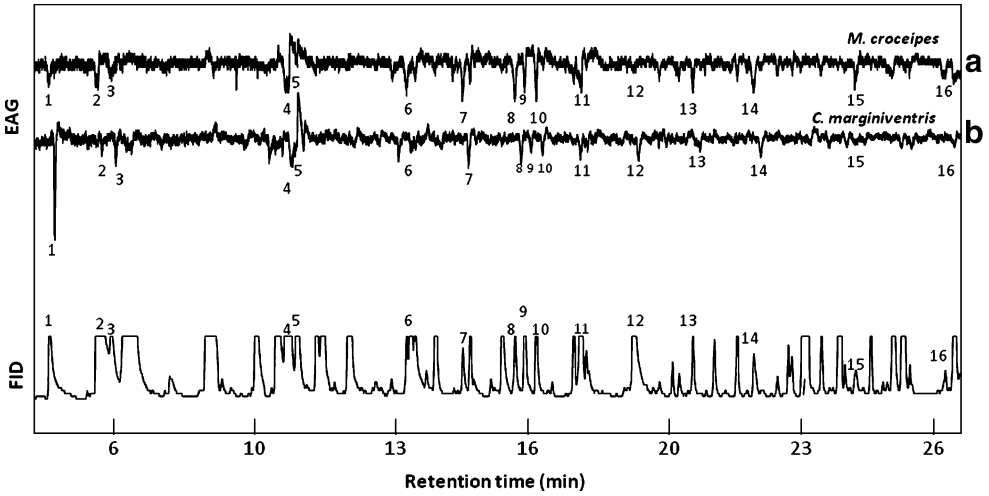

Fig. 2 GC-EAD responses of *Microplitis croceipes* (**a**) and *Cotesia marginiventris* (**b**) to headspace volatiles from *Heliothis virescens* damaged cotton plants. GC-EAD active compounds: (1) *cis*-3-hexenal; (2) *trans*-2-hexenal; (3) *cis*-3-hexen-1-ol; (4) *cis*-3-hexenyl acetate; (5) *trans* -2-hexenyl acetate; (6) linalool; (7) (*E*)-4,8-dimethyl-1,3,7-nonatriene; (8) unknown; (9) *cis*-3-hexenyl butyrate; (10) *trans*-2-hexenyl butyrate ; (11) *trans*-2-hexenyl-2-methyl butyrate; (12) indole; (13) *cis*-jasmone; (14) α-farnesene; (15) α-humulene; (16) *trans*-nerolidol. Note that responses of *C. marginiventris* to some of the HIPV components were almost too low to be detectable in this and the next two figures. GC-EAD responses of both species to the various compounds are quantified in Table 2

initial experiment with the synthetic blend were neither too high nor physiologically irrelevant.

Discussion

The results show that *M. croceipes* and *C. marginiventris* females were capable of responding antennally to many but not all of the caterpillar-induced cotton volatiles, with both parasitoid species showing differential electrophysiological responses to the different blend components. Compared to undamaged plants, cotton plants emitted detectable amounts of a wide range of volatiles, specifically 30 compounds, in response to damage by *H. virescens* or *S. exigua*. In general, our results are in agreement with those previously reported by other authors on the induction of cotton volatiles (Loughrin et al. 1994; McCall et al. 1994), but with some important differences. Loughrin et al. (1994) and McCall et al. (1994) reported 23 and 22 compounds, respectively, from the headspace of caterpillar-infested cotton plants, most of

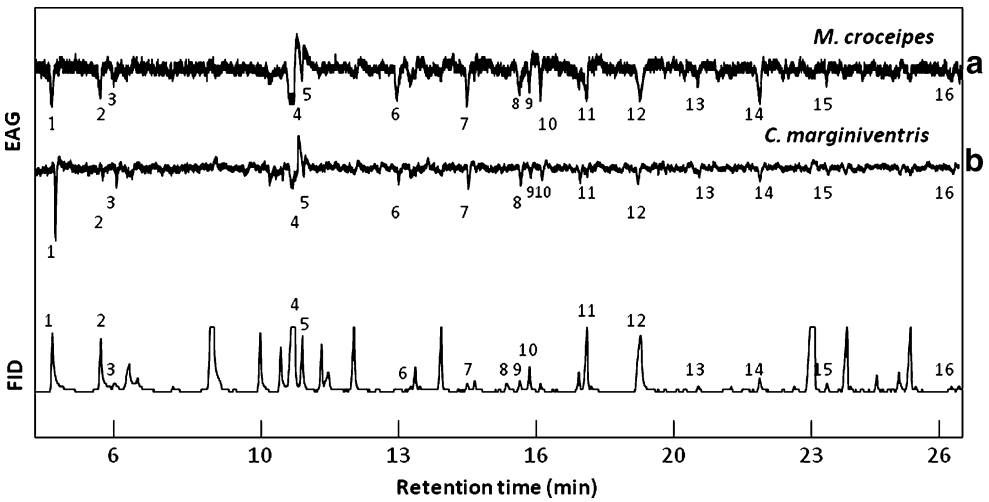

Fig. 3 GC-EAD responses of *Microplitis croceipes* (**a**) and *Cotesia marginiventris* (**b**) to headspace volatiles from *Spodoptera exigua* damaged cotton plants. GC-EAD active compounds: (1) *cis*-3-hexenal; (2) *trans*-2-hexenal; (3) *cis*-3-hexen-1-ol; (4) *cis*-3-hexenyl acetate; (5) *trans* -2-hexenyl acetate; (6) linalool; (7) (*E*)-4,8-dimethyl-1,3,7-nonatriene; (8) unknown; (9) *cis*-3-hexenyl butyrate; (10) *trans*-2-hexenyl butyrate; (11) *trans*-2-hexenyl-2-methyl butyrate; (12) indole; (13) *cis*-jasmone; (14) α-farnesene; (15) α-humulene; (16) *trans*-nerolidol. GC-EAD responses of both species to the various compounds are quantified in Table 2

Table 2 Quantification of GC-EAD responses of *Microplitis croceipes* and *Cotesia marginiventris* to the different components of headspace extracts of cotton plants infested with *Heliothis virescens* or *Spodoptera exigua*, and a synthetic blend of GC-EAD active components

ID	Compound[a]	*H. virescens*-infested		*S. exigua*-infested		Synthetic Blend	
		Microplitis croceipes (μV±SE)[b]	*Cotesia marginiventris* (μV±SE)[b]	*Microplitis croceipes* (μV±SE)[b]	*Cotesia marginiventris* (μV±SE)[b]	*Microplitis croceipes* (μV±SE)[b]	*Cotesia marginiventris* (μV±SE)[b]
1	*cis*-3-hexenal	72±6.6[b]	192±10[a]	56±4.0[b]	172±12[a]	140±8.9[b]	240±11[a]
2	*trans*-2-hexenal	64±6.3[b]	82±8.4a	56±4.0[b]	88±6.2[a]	62±4.8[b]	96±6.8[a]
3	*cis*-3-hexen-1-ol	44±4.0[b]	72±8.0[a]	48±8.0[b]	80±6.3[a]	76±4.5[b]	98±6.3[a]
4	*cis*-3-hexenyl acetate	144±7.2[a]	92±8.0[b]	176±6.4[a]	72±8.5[b]	136±7.4[a]	84±4.0[b]
5	*trans*-2-hexenyl acetate	52±6.3	48±6.3	54±6.3	46±5.8	96±7.4[a]	28±4.8[b]
6	linalool	72±6.9[a]	24±4.0[b]	80±6.3[a]	24±4.0[b]	80±7.4[a]	64±6.2[b]
7	4,8-dimethyl nonatriene	92±5.0	88±5.0	100±9.0[a]	44±4.0[b]		
8	unknown	108±5.0	88±8.0	100±12	72±4.8		
9	*cis*-3-hexenyl butyrate	104±7.5[a]	60±6.3[b]	172±8.0[a]	56±4.2[b]	240±10[a]	68±4.8[b]
10	*trans*-2-hexenyl butyrate	100±6.3[a]	60±5.3[b]	100±6.3[a]	32±4.8[b]	62±4.8[a]	28±3.6[b]
11	*trans*-2-hexenyl-2-methyl butyrate	60±6.3	40±8.9	88±8.0[a]	24±4.0[b]		
12	indole	24±9.8	36±7.5	80±6.3[a]	32±4.8[b]	28±4.8	16±4.0
13	*cis*-jasmone	52±4.8	38±4.8	48±5.8[a]	12±4.8[b]	88±4.8[a]	52±4.4[b]
14	α-farnesene	60±6.3	48±8.0	42±4.9	12±3.8	88±8.0[a]	24±4.0[b]
15	α-humulene	60±6.3[a]	8±3.8[b]	38±3.7	16±4.2	16±4.0	8±4.8
16	*trans*-nerolidol	16±4.0	12±4.8	12±4.8	9±4.8	20±6.3	20±6.3

[a] In order of elution during gas chromatography

[b] Values (μv) are mean±SE of five replicates

Means across the same row for the same headspace extract or synthetic blend followed by different letters are significantly different ($P<0.05$, *t*-test).

which were identified also in our study. These compounds included GLVs such as *cis*-3-hexenal, *trans*-2-hexenal, and *cis*-3-hexen-1-ol, and HIPVs such as *cis*-3-hexenyl acetate, linalool, (*E,E*)-4,8-dimethyl-1,3,7-nonatriene, *cis*-3-hexenyl butyrate, *trans*-2-hexenyl butyrate, *trans*-2-hexenyl-2-methyl butyrate, indole, *cis*-jasmone, (*E,E*)-α-farnesene, α-humulene, and *trans*-nerolidol. However, we detected additional volatile compounds that were not reported by Loughrin et al. (1994) and McCall et al. (1994), including *n*-decanal, (*E*)-2-hexenyl tiglate, and β-hemachelene. The differences

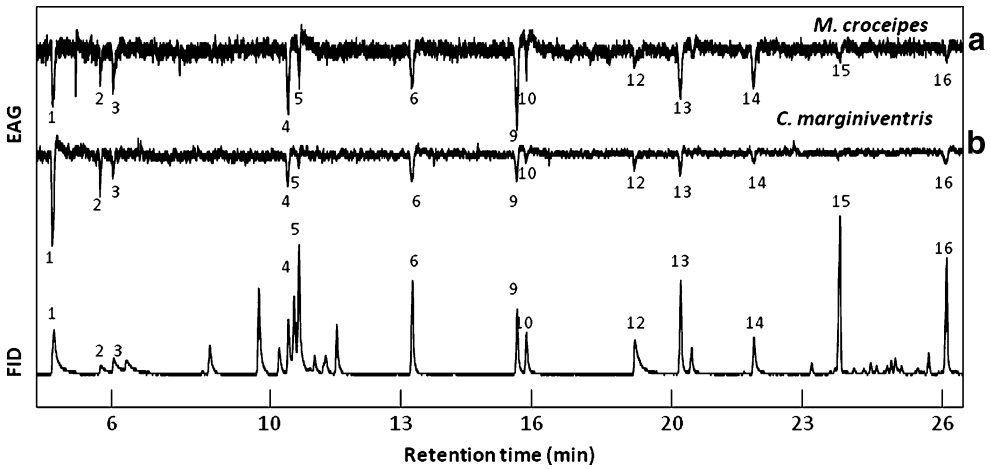

Fig. 4 GC-EAD responses of *Microplitis croceipes* (**a**) and *Cotesia marginiventris* (**b**) to a synthetic blend mimicking the headspace volatiles of caterpillar-infested cotton plants. The blend consisted of 13 compounds (listed below) identified as key biologically active components in the headspace volatiles of cotton plants infested with *Heliothis virescens*, and blended at an approximate ratio in which they were detected in the headspace. Synthetic compounds: (1) *cis*-3-hexenal; (2) *trans*-2-hexenal; (3) *cis*-3-hexen-1-ol; (4) *cis*-3-hexenyl acetate; (5) *trans* -2-hexenyl acetate; (6) linalool; (9) *cis*-3-hexenyl butyrate; (10) *trans*-2-hexenyl butyrate; (12) indole; (13) *cis*-jasmone; (14) α-farnesene; (15) α-humulene; (16) *trans*-nerolidol. GC-EAD responses of both species to the various compounds are quantified in Table 2

may be due to several factors, including differences in headspace volatile collection methodology, sensitivity of the analytical system, and cotton cultivar. For instance, we collected cotton volatiles continuously for 24 h beginning 12 h after plants were infested with caterpillars. Loughrin et al. (1994) collected volatiles for 3-h durations in each trap continuously for 60 h, beginning 1 h after plants were infested with caterpillars, while McCall et al. (1994) collected volatiles continuously for 2 h beginning 16–19 h after caterpillar feeding began. Furthermore, differences in the species/strains and stages of caterpillars tested may be important. Loughrin et al. (1994) used *S. exigua* caterpillars, while *H. zea* caterpillars were used by McCall et al. (1994). In the present study, we tested *H. virescens* and *S. exigua* caterpillars.

We recorded major differences in the amounts of volatiles induced by *H. virescens* vs. *S. exigua*. Of the total 30 components identified, 18 were detected in significantly higher amounts in the headspace of *H. virescens* damaged plants, compared to *S. exigua* damaged plants. These results suggest that the essential difference between the volatile blends induced by both caterpillar species is *quantitative*, rather than *qualitative*. Similar differences in the headspace volatile composition of plants infested by different herbivore species have been reported in cotton (McCall et al. 1994; Loughrin et al. 1994; De Moraes et al. 1998), corn (De Moraes et al. 1998; Turlings et al. 1998), cabbage (Agelopoulos and Keller 1994; Geervliet et al. 1997), and tobacco (De Moraes et al. 1998). It has been proposed that herbivore-specific volatile blends that differ significantly and consistently may provide reliable, information-rich signals to foraging parasitoids (De Moraes et al. 1998). Thus, the change in proportions or ratios of volatile compounds in the headspace of *H. virescens* damaged cotton plants, compared to *S. exigua* damaged plants may convey herbivore-specific information to specialist parasitoids, such as *M. croceipes*. On the other hand, generalist parasitoids, such as *C. marginiventris*, which have a wide host range, may not necessarily use herbivore-specific signals for host location. It is important to note that the use of plant volatiles by both parasitoids to locate host-infested plants may suggest that both are generalists in terms of host habitat location.

Only 16 of the 30 volatiles consistently elicited GC-EAD responses in *M. croceipes* and *C. marginiventris*, suggesting that not all components are perceived by both parasitoids, a finding in concert with those previously reported for some other parasitoid wasps (Li et al. 1992; Park et al. 2001; Smid et al. 2002; Gouinguené et al. 2005). The reason why parasitoids do not perceive all components of the headspace volatile of caterpillar-damaged plants is an interesting evolutionary question that deserves to be addressed. It is note worthy that most of the 16 GC-EAD active volatile compounds were among those elevated in *H. virescens* damaged plants. Our results showed no obvious *qualitative* differences in the range of compounds detected by either parasitoid species.

This is the first comparative study of GC-EAD responses of both parasitoid species to herbivore-induced cotton volatiles. In one of the few similar studies on other tritrophic systems, Smid et al. (2002) reported no differences in the GC-EAD responses of the specialist parasitoid, *C. rubecula* and the generalist, *C. glomerulata* to a wide range of volatiles from Brussels sprouts damaged by two species of *Pieris* caterpillars. In contrast, Gouinguené et al. (2005) reported some key differences in the GC-EAD responses of three parasitoid wasps to maize volatiles damaged by *Spodoptera littoralis* Boisduval caterpillars. Relatively more compounds elicited GC-EAD responses in the generalists, *C. marginiventris* and *Campoletis sonorensis* (Cameron), compared to *Microplitis rufiventris* Kok., which is found more often on *S. littoralis* (Gouinguené et al. 2005).

The major difference recorded in our study was in the intensity of GC-EAD response of both parasitoids to several compounds. We utilized a measurement tool in the GC-EAD software to quantify and then establish significant differences in GC-EAD responses of the two parasitoid species to the various volatile components. The generalist, *C. marginiventris* showed significantly greater GC-EAD responses than the specialist, *M. croceipes* to most GLV components, whereas several HIPV components elicited comparatively greater responses in *M. croceipes*. Similar differences in the intensity of response of parasitoids to host-related compounds also were reported by Gouinguené et al. (2005). The authors reported that the generalist parasitoids, *C. marginiventris* and *C. sonorensis*, showed a greater sensitivity to cotton GLVs *cis*-3-hexanal, *trans*-2-hexenal, and *cis*-3-hexen-1-ol) than the more restricted *M. rufiventris*. Our results in which females of the generalist *C. marginiventris* showed comparatively greater GC-EAD responses to GLVs (*cis*-3-hexenal, *trans*-2-hexenal, and *cis*-3-hexen-1-ol), which are continuously present in the plant and released in freshly damaged plants, support our hypothesis, and they are somewhat in agreement with previous electrophysiological (Gouinguené et al. 2005; Chen and Fadamiro 2007) and behavioral studies (Cortesero et al. 1997; Hoballah et al. 2002; D'Alessandro and Turlings 2005; Hoballah and Turlings 2005). Similar to our results, Gouinguené et al. (2005) also reported that *C. marginiventris* showed little or no antennal response to several HIPVs including β-myrcene, β-caryophyllene, bergamotene, and β-farnesene. In contrast, the specialist *M. croceipes* showed greater GC-EAD responses to the HIPVs, which are more specifically linked to its host. These findings were verified by the results of the GC-EAD tests with the synthetic blend, which also showed the same differences in the intensity of response of both parasitoids.

In general, *M. croceipes* showed slightly greater GC-EAD responses to headspace volatiles collected from cotton damaged by its host species (*H. virescens*) than to headspace volatiles collected from cotton that was damaged by the non-host species (*S. exigua*). Our GC data showed that the essential difference between the volatile blends induced by *H. virescens* vs. *S. exigua* is in the amounts and consequently the ratios of the same compounds. De Moraes et al. (1998) also reported that the main difference in the volatile blends of plants damaged by *H. virescens* vs. *H. zea* was in the ratios of identical compounds. Those authors further reported that the specialist parasitoid *C. nigriceps* could distinguish behaviorally plants damaged by its host, *H. virescens* from those damaged by *H. zea* (a non-host species), possibly by exploring the differences in the ratios of identical compounds in the volatile blends. Thus, the differences recorded in our study in the ratios of the same compounds in the blends induced by the two caterpillar species may be exploited by *M. croceipes* to differentiate plants damaged by its host from non-host species. This proposition is supported by our GC-EAD results which showed greater response of *M. croceipes* to volatiles from *H. virescens* damaged plants, compared to *S. exigua* damaged ones. The need to discriminate hosts from related non-hosts based on subtle differences in the ratios of identical compounds in blends is without doubt a challenging task for specialist parasitoids, such as *M. croceipes*. Thus, it is likely that other unknown minor compounds as well as host-specific volatiles also may play a role in differentiation of host vs. non-host by *M. croceipes*.

In contrast, no obvious differences were observed in the response of *C. marginiventris* to volatile blends induced by either caterpillar species. Our data for *C. marginiventris* are in agreement with the report by Geervliet et al. (1996) that a related generalist species, *C. glomerata* was unable to distinguish between plant volatiles induced by its hosts vs. plant volatiles induced by non-host species. However, *C. glomerata* was able to discriminate between plant volatiles induced by its hosts vs. volatiles induced by non-host species after learning (Geervliet et al. 1998). This suggests that associative learning may improve the overall ability of *C. marginiventris* to respond to the HIPV components of the volatile blends, as has been reported for some other generalist parasitoids (Turlings et al. 1989, 1993; Vet and Groenewold 1990; Vet 1999; Steidle and van Loon 2003; Tamo et al. 2006). Indeed, there is evidence that associative learning may improve response of *C. marginiventris* to induced volatiles (D'Alessandro and Turlings 2005). Furthermore, the results of an ongoing study in our laboratory suggest that associative learning may enhance the behavioral response of *C. marginiventris* to host-related volatiles (unpublished data).

The recorded differences in the antennal sensitivity of *M. croceipes* and *C. marginiventris* to host-related volatiles may be related to differences in the abundance and distribution of olfactory sensilla on the antennae of both parasitoid species. Sensilla placodea has been identified as the main olfactory sensilla responsive to host-related volatiles in *M. croceipes* (Ochieng et al. 2000) and *Cotesia* spp. (Bleeker et al. 2004). A comparative study of antennal morphology of the closely related *C. rubecula* and *C. glomerata* revealed significant differences in the density and distribution of this sensilla type (Bleeker et al. 2004). In an ongoing comparative study of antennal sensilla of *M. croceipes* and *C. marginiventris* in our laboratory, we recorded relatively greater numbers of olfactory sensilla placodea on *M. croceipes* than on *C. marginiventris* antennae (unpublished data). This difference in the density of olfactory sensilla may explain the differences in GC-EAD responses of both parasitoids recorded in this study.

In summary, the results may provide insight into how specialist parasitoids can distinguish between plants damaged by their hosts vs. plants damaged by closely related non-hosts, even though the different hosts may induce the emission of *qualitatively* similar volatile blends. The data suggest that differences between similar blends in the *ratios* of identical volatile compounds may contribute to host specificity in specialist parasitoids, such as *M. croceipes*. Additionally, unknown minor compounds as well as host-specific volatiles also may play a role in differentiation of different host-plant complexes. Further discrimination may be mediated at short range by host contact kairomones (which are typically of relatively lower volatility), such as host feces (Loke and Ashley 1984; Dmoch et al. 1985; Afsheen et al. 2008) and caterpillar chemical footprints on infested plants (Rostas and Wölfling 2009). Future behavioral studies are necessary to confirm whether or not the ability of *M. croceipes* to distinguish between plants damaged by its host and non-host caterpillars (Rosé et al. 1997), is in fact mediated by the subtle quantitative differences in volatile blends recorded in this study. If confirmed, the neurophysiological mechanisms that mediate this fine scale ability for odor discrimination will be addressed in the future by using single sensillum and neuroanatomical techniques.

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Here, we addressed induced indirect plant defense at different levels of biological integration. An example of an induced indirect defense mechanism is the production of volatiles by plants in response to herbivory. These volatiles are used by parasitoids or predators to locate their herbivorous victims. Herbivore-induced plant volatiles (HIPVs) mainly comprise green leaf volatiles (GLVs), terpenoids, and phenolics (Dudareva et al. 2006). The composition of induced volatile blends can vary qualitatively or quantitatively (Dicke and Hilker 2003). With this variation, the plant can provide the natural enemies of herbivores with detectable and reliable information (Vet and Dicke 1992; Dicke 1999). The induced volatile production is orchestrated by at least three main signal-transduction pathways: the jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) pathways (Dicke and Van Poecke 2002; Kessler and Baldwin 2002). These pathways can be differentially induced by different herbivore species (Heidel and Baldwin 2004; Schmidt et al. 2005; De Vos et al. 2005), leading to the emission of an herbivore-specific volatile blend (Vet and Dicke 1992; Ozawa et al. 2000; Walling 2000; Leitner et al. 2005).

Jasmonic acid (JA) is a member of a family of compounds collectively known as jasmonates or oxylipins, produced by the jasmonate pathway (Fig. 1). Leaf-feeding insects, such as the larvae of herbivorous Lepidoptera, especially induce the jasmonate pathway (Kessler and Baldwin 2002; De Vos et al. 2005). The production of jasmonates from linolenic acid (18:3) and linoleic acid (18:2) is initiated in the plastid and completed in the peroxisome and cytosol (Schaller et al. 2005). Lipases that release linolenic acid from membrane lipids, mainly originating from damaged cell walls, are thought to play an important role in regulating the response to herbivore-derived cues (Farmer and Ryan 1992; Schaller et al. 2005). Linolenic acid and linoleic acid subsequently are converted by lipoxygenase (LOX) (Bell et al. 1995), allene-oxide synthase (AOS) (Laudert and Weiler 1998), and allene-oxide cyclase (AOC) (Ziegler et al. 1997) into 12-oxophyto-dienoic acid (OPDA). A parallel cascade converts hexadecatrienoic acid (16:3) to dinor-oxophytodienoic acid (dnOPDA) (Weber et al. 1997). After the activity of 12-oxophytodienoic acid reductase (OPR) (Stintzi and Browse 2000) and three ß-oxidation steps, the oxylipins OPDA and dnOPDA are metabolized to form JA (Schaller et al. 2005). Another branch, starting at 9- or 13-hydroperoxide formed by the lipoxygenase, leads to the production of GLVs through the action of hydroperoxide lyase (HPL) (Bate et al. 1998).

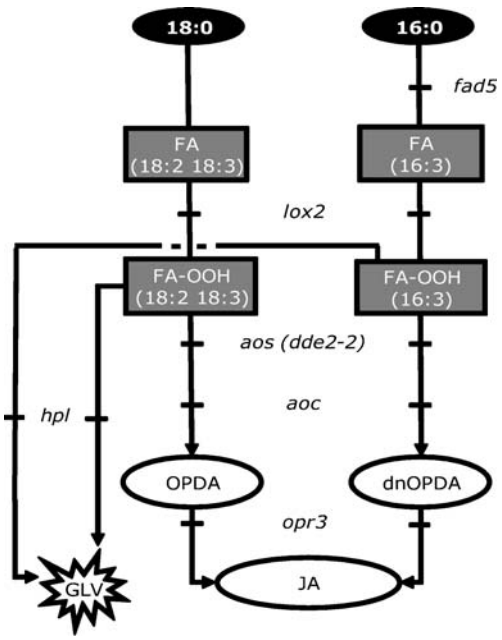

Fig. 1 Biosynthetic route of jasmonates in infested *Arabidopsis thaliana* leaves. *FA* Fatty Acid; 16:0 = hexadecanoic acid; 16:3=7Z, 10Z,13Z-hexadecatrienoic acid; 18:0 = octadecanoic acid; 18:2= 9Z,12Z-octadecadienoic acid (linoleic acid); 18:3=9Z,12Z,15Z-octadecatrienoic acid (linolenic acid); *dn-OPDA* dinor-oxo-phytodienoic acid; *OPDA* oxo-phytodienoic acid; *JA* jasmonic acid; *GLVs* green leaf volatiles. The HPL pathway also utilizes 9− and 13− hydroxides from hexadecanoic, linoleic and linolenic acid. Mutations affecting the biosynthesis are indicated in *italics*

Thus, herbivory results in a change in the oxylipin signature in a plant (Vollenweider et al. 2000). However, it remains to be unraveled what the relative contribution of the different oxylipins is in the induction of indirect defense in terms of HIPV emission and the attraction of natural enemies of herbivores. Evidence for the involvement of jasmonates in herbivore-induced responses does not stem only from the induction of JA and its intermediates upon herbivory. Exogenous application of jasmonates mimics the effects of herbivory. For example, exogenous application of methyl jasmonate (MeJA) and caterpillar-feeding induce in Arabidopsis similar, but not identical, transcriptional responses (Reymond et al. 2004). De Vos et al. (2005) demonstrated that there is roughly a 50% overlap in gene induction in Arabidopsis after MeJA treatment and herbivory by caterpillars or thrips. Besides MeJA, other jasmonates also trigger transcriptional changes. Interestingly, exogenous treatment with OPDA, JA, or MeJA results in overlapping but not identical gene-expression profiles in Arabidopsis (Taki et al. 2005).

Second, jasmonates also influence the emission of volatiles by plants. The production of volatiles by JA-treated plants is quantitatively and qualitatively similar compared to induction by herbivory in Lima bean (Dicke et al. 1999; Ozawa et al. 2000). Application of OPDA has effects on secondary metabolite production similar to applying MeJA, in cell cultures of several plant species (Gundlach and Zenk 1998). However, exogenously applied OPDA but not JA induces diterpenoids in Lima bean plants (Koch et al. 1999).

A third line of evidence for the involvement of jasmonates in herbivore-induced responses comes from mutant analyses. For example, Arabidopsis *opr3* and *aos* mutants show different gene-expression profiles in response to JA, MeJA, OPDA, and mechanical damage compared to wild-type plants, thus indicating distinct signaling roles for dnOPDA, OPDA and JA (Stintzi et al. 2001; Taki et al. 2005). Indeed, *opr3* mutants that lack JA still show oxylipin-dependent resistance to pathogens and herbivores, implying a role for jasmonates other than JA in plant defense (Stintzi et al. 2001).

Mutations in the oxylipin pathway also affect indirect defense. In *Nicotiana attenuata* for example, AOS-silenced plants (as-*aos*), display a reduced JA accumulation and terpenoid emission, and antisense-*hpl* mutants release fewer GLVs (Halitschke et al. 2004). Similarly, two antisense-*hpl*-mutants show an altered production of GLVs in Arabidopsis (Shiojiri et al. 2006a). These oxylipin-mediated effects on volatile emissions are accompanied by effects on interactions of plants with natural enemies of herbivores (Shiojiri et al. 2006b; Halitschke et al. 2008).

Thus, several lines of evidence demonstrate the involvement of jasmonic acid in herbivore-induced responses, including indirect defense, yet also suggest roles for other intermediates of the jasmonate pathway such as dnOPDA or OPDA. As predators and parasitoids are able to discriminate JA-induced from herbivore-induced volatiles (Dicke et al. 1999; Gols et al. 1999; Van Poecke and Dicke 2002), other signals besides JA are likely required for the induction of indirect defenses. Mutant plants altered in signaling pathways with a changed volatile emission represent some of the most powerful tools of testing the mechanisms that underlie HIPV production. The investigation of the effects of altered HIPV production in behavioral assays then is a valuable key to unravel the ecological relevance of these signaling pathways. Here, we followed a molecular ecological approach to study the involvement of several intermediates from the jasmonate pathway in the induction of plant volatiles by leaf-feeding herbivores. We used *Arabidopsis thaliana* to dissect the jasmonate pathway and analyze the effects on indirect defense after attack by leaf-feeding herbivores. In particular, we were interested in the contribution of the two sub-pathways that originate from galactolipids (16:0) or phospholipids (18:0) (Schaller et al. 2005), with special interest in dnOPDA, OPDA, and JA. For this, we selected mutants with altered production levels of dnOPDA, OPDA, and JA (Weber et al. 1997; Stintzi and Browse 2000; Stintzi et al. 2001; Von Malek et al. 2002). For caterpillar-infested mutants and their corresponding wild-type plants, the levels of dnOPDA, OPDA, and JA were quantified. Subsequently, HIPVs were collected, and volatile blend composition was quantitatively analyzed. Finally, we quantified caterpillar-feeding rate and conducted behavioral bioassays with parasitoid wasps to determine the effects of the observed differences in oxylipin profiles and HIPV blend composition after caterpillar-feeding on species interactions.

Methods and Materials

Plants and Insect Material Arabidopsis seeds (*A. thaliana*; genotypes Columbia (Col-0), Wassilewskija (WS), *fad5*, *opr3*, and *dde2-2*) were germinated on an autoclaved mixture of commercially available potting soil and 33% sand, and cultivated in a growth chamber at 21±2°C, 50 to 60% relative humidity (RH), and L8:D16h photoperiod with 80 to 110 μmol m^{-2} sec^{-1} PPF. The selected mutant *fad5* has a Col-0 background, and is incapable of biosynthesizing 7Z,10Z,13Z-hexadecatrienoic acid (16:3) (Weber et al. 1997); the mutant *dde2-2* also has a Col-0 background and is defective in allene oxide-synthase (AOS) (Von Malek et al. 2002). The mutant *opr3* has a WS background and lacks the most relevant isozyme of 12-oxo-phytodienoate reductase (OPR) (Schaller et al. 2000; Stintzi and Browse 2000; Stintzi et al. 2001). Two-wk-old seedlings were transferred to plastic cups (5 cm diam) filled with the earlier described soil mixture. Plants were watered twice a week. When plants were full-grown, vegetative plants, i.e., 6–8 wk after sowing, they were used for experiments.

Herbivore-induced defense responses were initiated by caterpillars from *Pieris rapae*, the small cabbage white. *Pieris rapae* was reared on Brussels sprouts plants (*Brassica oleracea* var. *gemmifera*, cv Cyrus) in a climatized room (L16:D8h; 20±2°C and 70% RH).

The parasitoid wasp *Diadegma semiclausum* was reared on *Plutella xylostella* caterpillars feeding on Brussels sprouts in a climatized room (L16:D8h; 20±2°C and 70% RH). Emerging wasp species were provided ad libitum with water and honey for 2–5 d until experiments were conducted, and are referred to as 'naïve' wasps, as they had received no exposure to plant material, nor an oviposition experience.

Plant Treatments Defense responses were induced by herbivore feeding, or by spraying the plant with JA. Plants were infested by equally distributing 20 first-instar *P. rapae* larvae over the fully expanded leaves. Herbivore feeding was mimicked by spraying JA. Four plants were sprayed with 5 ml of 1.0 mM (±)–JA (Sigma-Aldrich) aqueous solution. JA treatment was performed outside the climate room, for ca 15 min, under comparable climatic conditions. After treatment, plants were placed back into the climate room. In all experiments, plants were treated 24 h before the experiments and kept in a climate room (21±2°C, 50–60% RH; L8:D16h photoperiod and 80 to 110 μmol m^{-2} sec^{-1} PPFD).

Quantitative Analysis of Jasmonate Family Members The abundance of dnOPDA, OPDA, and JA, was determined for each of the used Arabidopsis genotypes. After 24 h, *P. rapae* larvae were removed. All rosette leaves from infested or uninfested control plants were harvested, immediately weighed, and frozen in liquid nitrogen for storage. Extraction of the oxylipins was performed according to the protocol described by Weber et al. (1997). For quantifying the derivatized oxylipins, a gas chromatograph (Hewlett–Packard 5890) equipped with a 30 m×0.25 mm HP-MS column (Hewlett–Packard) coupled to a mass-spectrometer (model 5972, Hewlett–Packard) was used. Helium was used as carrier gas with a flow of 1 ml min^{-1}. The column temperature at the moment of injection was 100°C. The temperature gradient was 100°C to 160°C at 20°C min^{-1}, 160°C to 238°C at 3°C min^{-1}, and 238°C to 300°C at 30°C min^{-1}. Quantification was done by measuring selective ions *m/z*=224 for methyl jasmonate, *m/z*=278 for methyl dnOPDA, *m/z*=238 for methyl OPDA, *m/z*=226 for methyl dihydrojasmonate (Internal Standard used for methyl JA), and *m/z*=240 for methyl tetrahydro-OPDA (Internal Standard used for methyl dnOPDA and methyl OPDA). Internal standards, methyl dihydrojasmonate and methyl tetrahydro-OPDA, were synthesized according to the protocol described by Weber et al. (1997).

Amounts of dnOPDA, OPDA, and JA were calculated per gram fresh weight. In cases where the oxylipin quantity was below the detection level for a certain sample, a value of 0.01 was assigned to these samples. Values were log10 transformed. The following fixed effects-model was used for each oxylipin to screen for differentiation per genotype: $\log 10(O_{ij}) \sim G_i + T_j + G : T_{ij} + \varepsilon_{ij}$, where O = oxylipin quantity per gram fresh weight; G = genotype; T = treatment; ε = residual; i=1,…,4; and j=1,2. Subsequently, per oxylipin two-tailed *t*-tests among Genotype:Treatment combinations were performed to obtain *P* values. For the 2004 data, the *t*-tests were followed by a Benjamini and Hochberg false discovery rate (BH-FDR) multiple comparison correction, to obtain the *q* values (Benjamini and Hochberg 1995). All linear models were performed in the R environment (R Development Core Team 2008) with R packages for linear mixed-effects models.

Headspace Collection and Volatile Analysis Dynamic headspace sampling was done for sets of 4 plants in a climate room (20±2°C, 70% RH; L8:D16h photoperiod and 90 to 110 µmol m^{-2} sec^{-1} PPFD). Twenty-four h before trapping, the pots were removed, soil with the roots were carefully wrapped in aluminum foil, and the 4 plants were placed together in 2.5-liter glass jar. Plants either were left uninfested or were infested with 20 first-instar (L1) *P. rapae* per plant. Just before trapping, jars were closed with a Viton-lined inert glass lid having an inlet and outlet. Air was sucked out with a vacuum pump at 100 ml min^{-1} with the incoming air purified through a steel cartridge filled with 200 mg Tenax-TA (20/35-mesh, Grace-Alltech, Deerfield, MI, USA). A same kind of cartridge was used to trap emitted plant volatiles at the outlet. After 3.5 h of trapping at continuous light, fresh weights of the 4 plants were measured. Headspace collections of uninfested and infested plants, for all the genotypes, were carried out in parallel on one experimental day.

Headspace samples were analyzed with a Thermo TraceGC Ultra™ (Thermo Fisher Scientific, Waltham, MA, USA) connected to a Thermo TraceDSQ quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Before desorption of the volatiles, the cartridges were dry-purged with helium at 30 ml min^{-1} for 20 min at ambient temperature to remove moisture. Samples were desorbed from the cartridges by using a thermal desorption system at 250°C for 3 min (Model Ultra Markes Llantrisant, UK) with a helium flow at 30 ml min^{-1}. Analytes were focused at 0°C on an electronically-cooled sorbent trap filled with Tenax and Carbograph (Unity™, Markes International LTD, Llantrisant, UK). Volatiles were transferred without split to the analytical column (Rtx 5MS, 30 m×0.25 mm i.d., 1.0 µm film thickness, Restek, Bellefonte, PA, USA) by ballistic heating of the cold trap to 300°C. The GC was held at an initial temperature of 40°C for 3.5 min followed by a linear thermal gradient of 10°C min^{-1} to 280°C and held for 2.5 min with a column flow of 1 ml min^{-1}. The column effluent was ionized by electron impact ionization at 70 eV. Mass spectra were acquired by scanning from 45 to 400 m/z with a scan rate of 3 scans sec^{-1}.

Compounds were identified by using the deconvolution software (AMDIS version 2.64, NIST, USA) in combination with NIST 98 and Wiley 7th edition spectral libraries and by comparing their retention indices with those from literature (Adams 1995). Characteristic quantifier ions were selected for each compound of interest. Metalign software (PRI-Rikilt, Wageningen, The Netherlands) was used to align chromatograms of all samples and integrate peak areas for the signals of the quantifier ions. Peak areas were converted to peak area per gram fresh weight of leaf material.

Areas of quantifier ions per gram fresh weight were log10 transformed, and for each HIPV compound the following mixed model was used to screen for HIPV compound differentiation per genotype: $\log 10(V_{ijk}) \sim G_i + T_j + G : T_{ij} + R_k + \varepsilon_{ijk}$, where V = area of quantifier ions per gram fresh weight; G = genotype; T = treatment; R = replicate; ε = residual; i=1,…,5; j=1,2; and k=1, …,5. Both G and T were used as fixed effects and R as a random effect. Subsequently, two-tailed *t*-tests followed by a Benjamini and Hochberg false discovery rate (BH-FDR) multiple comparison correction were conducted per compound for the genotypes (Benjamini and Hochberg 1995).

Similarities among plant genotypes based on volatile profiles were analyzed by using LEGG, a nonlinear dimensionality reduction method based on uncentered Pearson correlations among volatile profiles, which are used to generate 3-dimensional networks, for details see Van Poecke et al. (2007).

Behavioral Assays The effects of HIPV alterations as a result of mutations in the jasmonate pathway on behavioral responses were tested for the parasitoid species *Diadegma semiclausum*. Behavioral assays were carried out in a closed Y-tube olfactometer system as described in detail by Takabayashi and Dicke (1992). To investigate the behavioral responses of 3 to 7-d-old mated *D. semiclausum* females, a modified Y-tube olfactometer was used (Bukovinszky et al. 2005). In short, filtered air was led through activated charcoal and split into two air streams (4 l min^{-1}) that were led through 5-liter glass vessels containing the odor sources that consisted of 4 plants each. Plants were infested with 20 L1 *P. rapae* or sprayed with 1.0 mM JA solution 24 h before starting the bioassay. Plants were kept overnight in a climate room (21±2°C and 50 to 60% RH, L8:D16h photoperiod and 80 to 110 µmol m^{-2} sec^{-1} PPF). The olfactometer was illuminated with 4 high-frequency fluorescent tubes (Philips 840, 36 W) from above at an intensity of 60±5 µmol photons m^{-2} sec^{-1}. All experiments were conducted in a climatized room (20±2°C).

Individual wasps were transferred into the Y-tube olfactometer, and their behavior was observed and scored as described in detail by Bukovinszky et al. (2005). Odor sources were interchanged to compensate for any unforeseen asymmetry in the set-up after every 5 wasps tested. Choices between odor sources were statistically analyzed by using a chi-square test, with the null-hypothesis that no preference existed.

The total area of consumed leaf-tissue was analyzed for the plants used in the bioassay. Therefore, after an experiment, all individual leaves of each rosette were taped on paper and scanned with a Hewlett–Packard scan jet 3570c. For quantification of the consumed leaf area, analysis was performed with KS400 version 3.0 software service pack 9 (Carl Zeiss Vision, Oberkochen, Germany). The consumed leaf area per genotype was statistically compared by using ANOVA (SPSS 15.0, Chicago, IL, USA).

Results

Quantitative Analysis of Jasmonate Family Members We quantified the levels of dnOPDA, OPDA, and JA in leaf-tissue from the mutants *dde2-2*, *opr3*, and *fad5*, after 24 h of herbivory by *Pieris rapae*, and from uninfested plants (Fig. 2, Supplementary Table 1). As the *dde2-2* and *fad5* mutants have a Col-0 background, while the *opr3* mutant has a WS background, both wild-type accessions were included as well.

Analyses of Col-0 were performed both in 2004 and 2005. Even though plants were grown in a controlled environment, the levels of oxylipins varied between the years (Fig. 2). Therefore, the 2004 dataset was analyzed separately from the 2005 dataset. Constitutive levels of OPDA and JA are

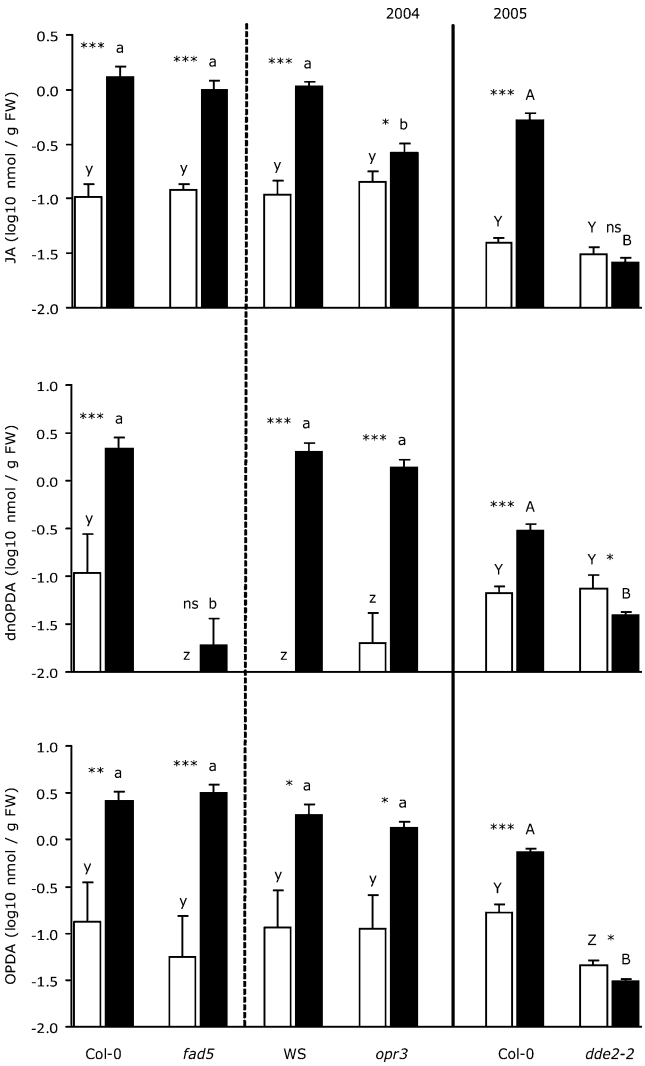

Fig. 2 Oxylipin amounts in *Arabidopsis thaliana* after 24 h of *Pieris rapae* feeding. Extracted oxylipin concentration for *P. rapae*-infested (*black bar*) or uninfested (*white bar*) leaves. Mean oxylipin values + SE are shown (N=4, see also Supplementary Table 1). *Dashed line* separates wild-type accession backgrounds for 2004. *Asterisks* indicate significant differences between infested and uninfested plants within a genotype (*P<0.05; **P<0.01; ***P<0.001; *ns* not significantly different). Bars for infested leaves marked with the same letter are not significantly different (data from years 2004 and 2005 were analyzed independently, for the 2004 data we used *lower case letters* and for data from 2005 *capitals*) (2004: q value >0.05; 2005: P value >0.05). dnOPDA amounts in undamaged *fad5* and WS plants were below detection limit

similar in both wild-type accessions, whereas constitutive dnOPDA levels are lower in WS compared to Col-0 (Fig. 2). Herbivory by *P. rapae* induced all three oxylipins to similar levels in Col-0 and WS plants (Fig. 2).

Mutations in oxylipin biosynthetic genes clearly affected the oxylipin signatures. In *dde2-2* plants, induction of dnOPDA, OPDA, or JA by herbivory was completely abolished. In fact, a decrease in dnOPDA and OPDA levels was observed in *dde2-2* plants in response to herbivory. Moreover, constitutive OPDA levels were lower in the *dde2-2* mutant.

A mutation in *FAD5* resulted in constitutively lower dnOPDA levels that could not be induced by herbivory. However, this mutation did not affect either constitutive or herbivore-induced levels of OPDA or JA.

A mutation in *OPR3* hampered the induction of JA after herbivory: herbivory resulted in significantly lower JA levels compared to the WS wild-type. Constitutive levels of all three oxylipins were unaffected by *OPR3* mutation.

In short, the oxylipin mutations have the expected effects on oxylipin production: the *fad5* mutant only affects dnOPDA levels, *opr3* only affects JA levels, and *dde2-2* affects the levels of dnOPDA, OPDA, and JA. Thus, these three mutants allow us to dissect the roles of dnOPDA, OPDA, and JA levels in indirect defense.

Volatile Analysis To assess the effects of altered oxylipin signatures on HIPVs, we measured the volatile emissions in uninfested and *P. rapae*-infested Col-0, WS, *dde2-2, fad5*, and *opr3* plants (Supplementary Table 2). Compounds for which an influence on discriminative behavior by carnivorous arthropods was known were selected and presented in Fig. 3 (De Boer et al. 2004; Dicke et al. 1990b; Shimoda et al. 2005). HIPV production in wild-type Col-0 and WS showed both similarities and differences. For example, *P. rapae* feeding induced the emission of methyl salicylate (MeSA), the sesquiterpene (*E,E*)-α-farnesene, and the homoterpene (*E,E*) 4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) to similar levels in both accessions. However, Col-0, but not WS, showed the induction of the monoterpene linalool, whereas WS, but not Col-0, showed induction of the GLVs (*Z*)-3-hexenal and (*Z*)-3-hexen-1-ol acetate, and of the monoterpenes β-myrcene and both stereoisomers of β-ocimene.

A mutation in *FAD5* did not result in an altered HIPV emission for the analyzed compounds, thus indicating that dnOPDA does not play a role in HIPV induction. In contrast, a mutation in *DDE2-2* abolished the induction of all analyzed HIPVs, except for 1-nonanol. A mutation in *OPR3* blocked induction of all analyzed HIPVs, except for the GLVs.

To compare total volatile blends instead of single compounds among accessions, the variation in volatile profiles among genotypes was explored further by using the algorithm locally linear embedding graph generator (LEGG). LEGG analysis resulted in a 2D network of genotypes, generated by using a non-linear dimensionality

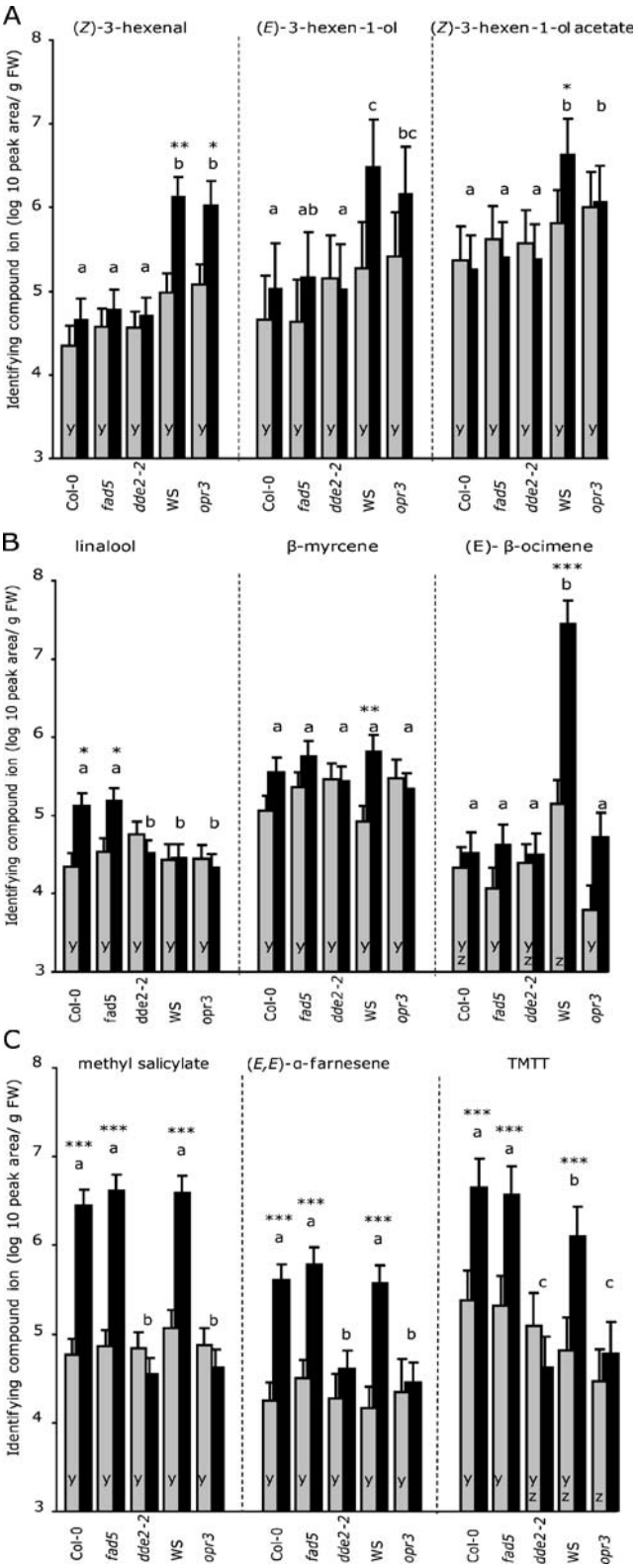

Fig. 3 Characteristic *Arabidopsis thaliana* HIPV-compounds per genotype treatment. **a**: GLVs; **b**: monoterpenes **c**: methyl salicylate (MeSA), (*E,E*)-α-farnesene, and (*E,E*) 4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT). Given is the mean + SE for compound-representative ions (N=4–5 replicates per treatment, see also Supplementary Table 2). *Asterisks* indicate significant differences between uninfested (*grey bars*) and *Pieris rapae*-infested (*black bars*) leaves within a genotype (*P< 0.05; **P<0.01; ***P<0.001). Bars that are marked with the same letter are not significantly different, (y–z = uninfested plants, a–c = infested plants), q value >0.05. *Dashed lines* divide separately analyzed compounds

reduction method (Fig. 4) (for LEGG details see Van Poecke et al. (2007) and the legend of Fig. 4). This demonstrated a strong relationship between volatile profiles emitted by uninfested plants of the various genotypes plus caterpillar-infested plants of the *dde2-2* and *opr3* mutants. In contrast, HIPVs from caterpillar-infested Col-0, *fad5*, and WS showed a much weaker correlation with the volatile blend from uninfested genotypes. LEGG analysis also showed that the HIPVs from Col-0 and WS show similarities.

Thus, headspace analysis for the mutants *fad5*, *dde2-2*, *opr3*, and their respective wild-types pointed out that only hampered JA levels (i.e., the common denominator in *dde2-2* and *opr3* plants) resulted in an altered production of HIPVs.

Behavioral Assays To investigate whether the changes in HIPV profiles due to the mutations in the jasmonate pathway affect indirect defense, we investigated the behavioral responses of a parasitoid wasp species towards HIPVs originating from *dde2-2*, *opr3, fad5*, and their wild-types in a Y-tube olfactometer (Fig. 5). *Diadegma semiclausum* females preferred volatiles of infested plants over uninfested plants for all mutants or wild-types (P<0.001). However, HIPVs from caterpillar-infested wild-type plants were significantly more attractive than HIPVs from caterpillar-infested mutants *dde2-2* or *opr3* (for both mutants P<0.05). The wasps did not discriminate between volatiles of herbivore-infested *fad5* plants and infested wild-type plants.

A possible explanation for the observed differences in HIPV emission and parasitoid attraction among mutants and wild-type plants is that the mutation altered the feeding behavior of the herbivore. A reduced attraction to infested mutant plants might have resulted from a reduced feeding rate on the mutant plants. However, this is not supported by our data on feeding rates: the amount of leaf area consumed did not differ when the caterpillars were feeding on plants of *opr3*, *fad5*, or their wild-types WS and Col-0 (308±21.6, 285±15.8, 284±18.1, 295±17.7 mean mm^2 ± SE / plant, respectively; P>0.05). Moreover, the caterpillars even consumed more leaf material of *dde2-2* plants compared to wild-type Col-0 (211±12.4 and 169±10.8 mean mm^2 ± SE / plant,

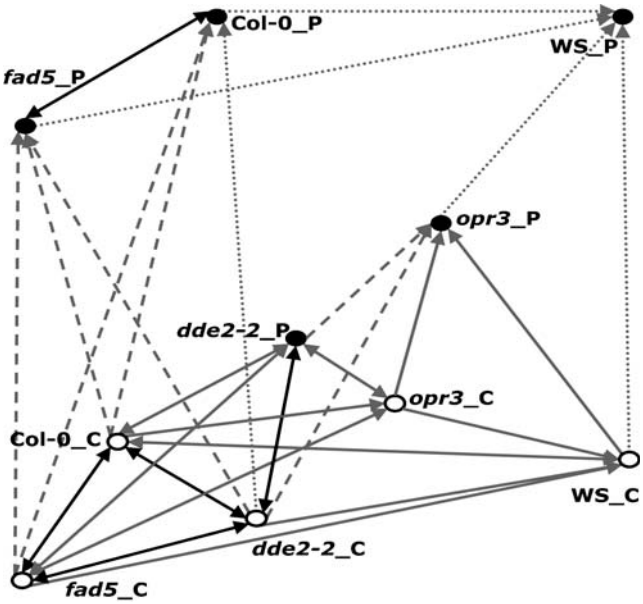

Fig. 4 Visualization of the relationships among HIPV blends of different *Arabidopsis thaliana* genotype-treatment combinations. To assess the similarities among volatile blends from different genotype-treatment combinations, LEGG analysis was applied to the volatile blends of the *Pieris rapae*-infested (_P ; black nodes) and uninfested (_C; white nodes) plants of different genotypes. Similarities were calculated as uncentered Pearson correlation coefficients, based on the quantitative information on 22 volatile compounds for each genotype treatment combination (1-pentanol; (*E*)-2-penten-1-ol; hexanal; (*Z*)-3-hexenal; (*E*)-3-hexen-1-ol; 1-hexanol; heptanal; α-pinene; 1-octen-3-ol; 6-methyl-5-hepten-2-one; β-myrcene; (*Z*)-3-hexen-1-ol acetate; (*Z*)-β-ocimene; (*E*)-β-ocimene; (*E*)-2-nonen-1-ol; linalool; (*E*)-4,8-dimethyl-1,3,7-nonatriene; 1-nonanol; methyl salicylate; undecanal; (*E,E*)-α-farnesene; (*E,E*) 4,8,12-trimethyl-1,3,7,11-tridecatetraene). The similarities were visualized using non-linear dimensionality reduction. Similarities among the profiles that were determined by LEGG are depicted as directed links. Similarities can be compared based on the presence, absence or strength of the directed links. The strength of the similarities is indicated by shading and line-type. Arrow lengths do not illustrate strength of connections. Thus, *solid black arrows* connect blends from genotype-treatment combinations that are highly similar (0.984<r^2<0.990). This similarity decreases from *solid grey arrows* (0.974<r^2<0.984); *dashed grey arrows* (0.964<r^2<0.974); to *dotted grey arrows* (0.951<r^2<0.964). Blends from genotype-treatment combinations that are not connected are relatively dissimilar. A genotype-treatment combination at the base of an arrow represents a near neighbor of the genotype-treatment combination directed at. Arrows that point both ways represent genotypes-treatment combinations that are both near neighbors of each other. For example, the volatile blend from *P. rapae*-infested *dde2-2* mutants is highly similar to the blend from uninfested *dde2-2* and other uninfested genotypes (nearest neighbours both ways, with relatively strong connections), whereas the blend of *P. rapae* infested Col-0 is quite dissimilar from uninfested Col-0 (uninfested Col-0 is a nearest neighbour of infested Col-0, but not the other way around, and the connection is relatively weak). In short, LEGG provides a relatively easy visual interpretation of similarities among volatile blends based on all 22 components

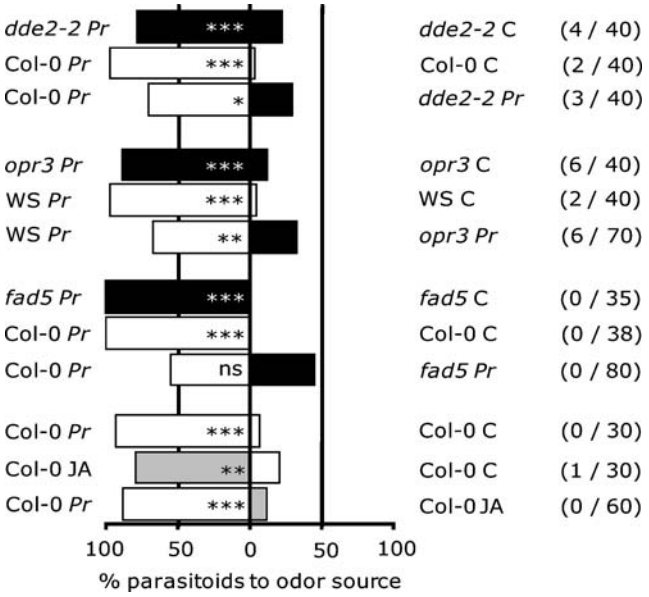

Fig. 5 Preference of naïve *Diadegma semiclausum* females to volatiles of differently treated *Arabidopsis thaliana*, as assessed in the Y-tube olfactometer. Plants were infested with *Pieris rapae* (*Pr*) or treated with JA 24 h before (JA) or were left untreated (C). *Data* represent total number of parasitoids that chose for any of the two odor sources as determined in 3–4 replicate experiments, each on a different day with new odor sources. Asterisks indicate a significant difference within a choice test: **P*<0.05; ***P*<0.01; ****P*<0.001; *ns* not significantly different (χ^2-*test*). The number of wasps that did not make a choice and the total number of tested parasitoids is given in *parenthesis*

respectively; $P<0.05$). Thus, the mutations do not result in reduced feeding and, thus, altered caterpillar-feeding rates cannot explain the observed changes in headspace composition or parasitoid attraction.

We verified the importance of JA signaling in indirect defense by assessing the attractiveness of volatiles induced in the plants by the application of 1 mM JA. Exogenous JA treatment of Col-0 plants made them more attractive than non-treated Col-0 control plants (Fig. 5, $P<0.01$). However, when these JA-sprayed plants were tested against *P. rapae*-induced Col-0, the wasps preferred the latter ($P<0.001$).

Discussion

The jasmonate pathway plays a pivotal role in the induced plant defense response against many arthropod herbivores, including indirect defense responses. However, the role of the different (intermediate) products of this pathway in these defense responses is unclear. Even though it was shown recently that JA needs to be conjugated to an amino acid for functionality, and that OPDA does not serve as a substrate for the conjugating enzyme, there are several reports that indicate a distinct role of OPDA in direct and indirect defenses, possibly through its electrophile properties (Ribot et al. 2008). Therefore, we set out to unravel the involvement of the jasmonate pathway products dnOPDA, OPDA, and JA in caterpillar-induced indirect defense by using a molecular ecological approach. We selected *Arabidopsis thaliana* mutants, *fad5*, *opr3*, and *dde2-2*, for which we quantified oxylipin levels, HIPV emission, and ultimately assessed parasitoid attractiveness towards HIPVs.

Based on previous work, summarized in Fig. 1, we expected the *fad5* mutant to show reduced levels of 16:3 derived compounds; the *dde2-2* mutant to show reduced levels of both 16:3 and 18:3 derived compounds; and *opr3* mutants to show reduced levels of JA. Our data confirmed these expectations: after herbivory *fad5* showed no induction of dnOPDA, *opr3* was hampered in the induction of JA, and *dde2-2* showed no induction of dnOPDA, OPDA, and JA (Fig. 2). Additionally, these data also demonstrated that 1) the 16:3 pathway does not contribute to constitutive or herbivory-induced levels of JA, and 2) that herbivory-induced levels of dnOPDA and OPDA do not depend on JA accumulation through a feedback loop.

Mutation of the *FAD5* gene did not result in an altered emission of HIPVs, showing that dnOPDA does not play a role in HIPV-mediated indirect defense. On the other hand, mutations in *DDE2-2* or *OPR3* did show clear effects on HIPV production, resulting in reduced induction of many volatiles, especially the terpenoids, thus indicating roles of OPDA and/or JA in HIPV-indirect defense. As *dde2-2* (which lacks both OPDA and JA) or *opr3* (which lacks only JA) show very similar HIPV production, we conclude that JA, and not OPDA, is the most important oxylipin in HIPV production. A minor role for OPDA cannot be excluded, as *opr3* plants, showing moderate JA induction, still showed induction of a few volatile compounds, such as GLVs, while *dde2-2* plants, lacking OPDA and JA induction, did not. However, it is likely that this difference between *opr3* and *dde2-2* is caused by differences in the genetic background: none of the three genotypes with a Col-0 background (Col-0, *fad5*, and *dde2-2*) showed induction of GLVs, whereas WS did. The lower GLV-levels produced by Col-0, *fad5*, and *dde2-2* plants are most likely caused by a dysfunctional HPL1 enzyme in the Col-0 background (Duan et al. 2005). In any case, the role of OPDA or earlier intermediates is minor, as LEGG analyses show that the composition of the overall volatile blend of *opr3* and *dde2-2* plants is similar (Fig. 4). Moreover, the blends of infested *dde2-2* and *opr3* plants were similar to the blends of uninfested controls, while they were different from the blends of infested wild-type plants (Fig. 4).

Parasitoid behavioral assays reflected the results obtained by headspace analyses: mutation of *FAD5* did not affect parasitoid behavior, whereas infested plants of both *opr3* and *dde2-2* showed reduced parasitoid attraction

compared to wild-type plants (Fig. 5). Some compounds were still induced in the mutants *dde2-2* and *opr3*: 1-nonanol and (*Z*)-3-hexenal, respectively (Supplementary Table 2, Fig. 3). These compounds might explain the attractiveness of infested mutants *dde2-2* and *opr3* over uninfested controls. These results indicate that other signals besides JA or its intermediates are involved in HIPV production. This is illustrated also by the observation that JA-treated Col-0 plants were less attractive to the wasps than caterpillar-infested Col-0 (Fig. 5; see also Van Poecke and Dicke (2002)). Similar results also have been obtained for other plant-herbivore-carnivore systems (Bruinsma et al. 2009; Dicke et al. 1999).

The combination of biochemical and behavioral analyses of Arabidopsis wild-type and mutant plants not only gives insight into which plant hormones influence indirect defense, but also through which volatile compounds these hormones exert their effects. Inhibition of JA production influenced mono-, sesqui-, and homoterpene volatile emissions as well as the emission of the shikimate pathway-derived methyl salicylate (MeSA). Even though exogenous JA is known to induce GLV production in Arabidopsis (Van Poecke 2002), we did not find any effect of the lack of JA on *P. rapae*-induced GLV emissions in Arabidopsis.

The importance of monoterpenes in plant-insect interactions is well studied (Dicke et al. 1990a; Rose et al. 1998; Shimoda et al. 2005; Mumm et al. 2008; Opitz et al. 2008). Even though Col-0 and WS differed in the identity of monoterpenes induced by *P. rapae* feeding, with Col-0 showing mainly induction of linalool and WS showing mainly induction of (*Z*)- and (*E*)-β-ocimene, lack of JA had a similar inhibitory effect on monoterpene induction in both accessions. Lack of JA also affected emission rates of the sesquiterpene (*E,E*)-α-farnesene and the diterpene-derivative (*E,E*) 4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT), demonstrating that JA influences not only the plastid-localized mono- and diterpene production, but also the sesquiterpene production located in the cytosol. Both (*E,E*)-α-farnesene and TMTT are known to influence plant-insect interactions (Van Poecke 2002; De Boer et al. 2004; Ibrahim et al. 2005).

Besides terpenoids, the induction of MeSA also was impaired in JA-lacking mutants. This indicates that hampering the JA pathway consequently impedes some step in the biosynthesis of MeSA. Previous studies using Arabidopsis demonstrated that blocking the SA pathway affects the emission of both MeSA and TMTT and results in decreased parasitoid attraction (Van Poecke and Dicke 2002; Van Poecke 2002). Thus, both JA and SA are required for herbivore-induced emissions of MeSA and TMTT. Such synergism also has been recorded for spider-mite induced volatile emissions of tomato plants (Ament et al. 2004).

In summary, the present study has shown the value of using a molecular ecological approach for elucidating the relative importance of jasmonic acid and its intermediates in indirect defense. By dissecting the jasmonate pathway through the use of selected mutants, we have gained a better understanding of the ecological role of oxylipins in HIPV-mediated indirect plant defense. Even though the importance of the octadecanoid pathway in indirect defense had been demonstrated previously (Thaler et al. 2002; Halitschke et al. 2004; Kessler et al. 2004; Shiojiri et al. 2006a, b), the relative contribution of the different oxylipin compounds to indirect defense had remained unclear. Here, we demonstrated that in Arabidopsis the 16:0 branch of the oxylipin pathway does not play a role in HIPV-mediated indirect defense. Moreover, we demonstrated that from the 18:0 pathway, jasmonic acid is the main actor in HIPV-mediated indirect defense, with limited or no contribution from pathway intermediates such as OPDA. The data strengthen the value of using a molecular ecological approach in advancing our understanding of multi-trophic plant-insect interactions.

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given host plant move within the plant to find adequate sources of food (Johnson and Zalucki 2005). They sometimes need to abandon their host and select another plant, for example when the plant resource is exhausted (Johnson and Zalucki 2005), when the competition from other herbivores becomes too strong (Pats and Ekbom 1992), when the plants' defense affects their health (Kester et al. 2002), following infection by a disease (Evans and Allaway 1983), or because of predation pressure (Bernays 1997). When they leave the host plant, their most relevant choice is to find a similar host to which they have adapted (Jermy et al. 1967; de Boer 2004), sparing the need to readjust their metabolism to a new food source. Actually, habituation to a host plant involves a number of changes, that include physiological adaptations, for example of taste perception (del Campo et al. 2001; Renwick 2001) as well as learning (Saxena and Schoonhoven 1978; Carlsson et al. 1999).

From an evolutionary point of view, it would make sense that larvae would express innate preferences for stimuli from suitable host plants and innate avoidance of stimuli from non-host plants. This holds true at least partly for contact chemoreception where taste neurons are tuned either to phagostimulant molecules or to potentially noxious compounds that are deterrent (Chapman 2003). Olfaction represents a different challenge, because plant odors are complex and variable, composed of ubiquitous chemicals such as green odors (Visser 1986), which makes any recognition task less easy. Larvae are equipped with olfactory sensilla located on their antennae and on the maxillary palps, representing approximately 78 neurons (Dethier and Schoonhoven 1969) that project into the antennal lobe (Kent and Hildebrand 1987; Itagaki and Hildebrand 1990). Electrophysiological studies confirm that some of these neurons detect plant odors, on the antenna (Dethier and Schoonhoven 1969; Dethier 1980) and on the maxillary palps (Roessingh et al. 2007). Despite this limited sensory equipment, neonate larvae of several Lepidoptera species are able to orient towards odors released from their host plants and to express specific choices (Huang and Mack 2002; Singh and Mullick 2002; Castrejon et al. 2006), presumably by recognizing patterns of combined odors and contact chemicals (Dethier 1976).

In this work, we used neonate larvae of the European corn borer (ECB), *Ostrinia nubilalis* Hübner (Lepidoptera: Crambinae) and tested their innate preferences towards odors released from a host and a non-host plant, respectively maize and spinach. ECB is considered a highly polyphagous species that has been reported on over 200 plant species (Hodgson 1928; Jackson et al. 2005 and references therein). Most adult females lay their eggs on maize (Bethenod et al. 2005; Bengtsson et al. 2006). They prefer maize over hop (*Humulus lupulus* L.), which is considered the ancestral host in Europe (Pelozuelo et al. 2004; Malausa et al. 2008). Recent molecular data suggest the species is composed of sympatric strains that exploit different host plants. In France, two strains coexist: a maize strain, which damages maize crops (used in this study) and a second strain, which oviposits and develops preferentially on hop and mugwort (*Artemisia vulgaris* L.) (Bethenod et al. 2005; Malausa et al. 2005; Leniaud et al. 2006).

Even if ECB larvae are polyphagous, they probably avoid feeding on a number of non-host plants that emit repellent or deterrent chemicals. One of the plants that ECB larvae and adults seem to avoid is spinach (*Spinacea oleraceae* L.). Spinach produces phytoecdysteroids (PEs) that act as a constitutive defense (Schmelz et al. 2000) and increases their synthesis and transport in response to damage from herbivores (Schmelz et al. 1998, 1999; Soriano et al. 2004). In the laboratory, ECB larvae avoid feeding on spinach, especially on younger leaves (F. Marion-Poll, unpubl. observations), which happen to have the richest content in PEs (Schmelz et al. 1999). ECB larvae also avoid feeding on a diet containing PEs such as 20-hydroxyecdysone (20E), which they detect with contact chemoreceptor neurons located in several of their taste sensilla (Marion-Poll and Descoins 2002). Deterrent cells with a similar sensitivity profile also are present in tarsal sensilla of the adults, and females avoid laying eggs when PEs are present at the surface of maize (Calas et al. 2007). PEs are generally toxic to insects because they interfere with ecdysone signaling (Dinan 2001). Although ECB larvae can detoxify PEs by producing fatty acyl ester conjugates (Rharrabe et al. 2007), we observed that larvae avoid feeding on a diet that contains PEs. Since ECB larvae and adults avoid PEs, we wondered if ECB larvae would be deterred by odorants emitted by spinach.

In this study, we monitored the orientation behavior of neonate ECB larvae stimulated with volatiles from maize or from spinach by using a locomotion compensator. Considering previous data, we expected larvae to orient towards maize odors and possibly walk away from spinach odors. Odors were sampled by head space entrainment with humidified air forced through a plastic bag that enclosed the leaves of a plant. The stimulus concentration was adjusted by changing the amount of air flowing through the bag. We also delivered odors at different pulsing regimes because the temporal structure of the odor plume has been shown to be of special importance to the orientation of adult moths towards pheromone or plant volatiles (Cardé and Willis 2008). Odor pulsing also has been recently reported to influence orientation of larvae of the Colorado potato beetle towards host-plant odors (Hammock et al. 2007). Our results demonstrate that ECB larvae showed a slight positive anemotaxis or did not orient to odorless humidified air stream, whatever the pulsing conditions. Conversely, they oriented spontaneously to odors emitted

by maize and spinach. At a stimulus stream of 40 ml/min, ECB larvae avoided spinach odors at odor pulses of 2 sec or lower pulse regimes. However, ECB larvae were attracted or repelled by maize odors depending on the stimulus stream dilutions (20, 40, or 60 ml/min) and their temporal pattern. Intermittent odor stimuli were found to elicit upwind orientation towards maize odor, while continuous or slow-pulsing stimuli induced a reversal of the orientation that is maintained over gaps of odorant stimuli of up to 10 sec.

Methods and Materials

Plant Culture 'Centena' maize were grown in individual pots in a greenhouse at 22±2°C during daytime and 18±2°C overnight, with a 16 L:8D photoperiod. Spinach 'Géant d'hiver' potted plants were grown in a climatic chamber at around 15±2°C day and night. Maize plants were watered four times weekly, and fertilized with Peters® General Purpose Fertilizer (J. R. Peters Inc., Allentown, PA, USA) at 100 ppm in aqueous solution twice each week as part of the regular watering schedule. Maize was used when it had developed 7 leaves (about 80 cm high). Spinach was used when it had grown 17 leaves (about 12 cm high). These experiments were performed in 2005 and 2006.

Insects ECB pupae were sent from INRA (Le Magneraud, France), where a culture is maintained on an artificial diet. Emerging adults were maintained in a cage at 25±0.5°C and 70–80% R.H. and fed sugar water *ad libitum*. Eggs were collected from a wetted filter paper placed on the top of the cage. Individual egg clusters were stored in glass vials (12 mm ID; 7.5 mm-long). Each vial was sealed with a cotton plug that was lightly wetted daily with distilled water to prevent desiccation. Upon hatching, larvae were starved 6–12 h prior to the experiments. Pilot observations indicated that this starving phase was necessary to obtain reproducible results.

Locomotion Compensator Experiments were performed with a locomotion compensator, TrackSphere LC-100 (Syntech, Hilversum, NL). This device is composed of a white sphere (100 mm diam) coupled to two step-motors driven by a controller (Van Tilborg et al. 2003). A video-camera inserted in the light path of a stereomicroscope monitored the position of the insect and the controller drove the step-motors so that the image of the insect remained in the center of the visual field. The position changes (dx_i, dy_i) of the insect were collected at a rate of 10 Hz, and the insect track was calculated and displayed on a PC screen under the control of the tracking software TrackSphere™ v 3.1 (Syntech, Hilversum, NL).

Stimulus Delivery A constant stream of humidified and charcoal-filtered air at 80 ml/min was supplied through an aluminum tube (1.6 cm ID, 27 cm-long; outlet: 3 cm from the top of the sphere) directed horizontally towards the top of the sphere. The airflow was controlled with a programmable controller (CS-55, Syntech, DE). An adjustable fraction of the airstream could be diverted either to a Nalophane® bag that enclosed a plant (stimulus), or to an empty tube (blank) by using a three-way electronic valve. The outflow of each of these streams was re-injected into the mainstream within the aluminum tube in order to maintain a constant flow. For maize, the stimulus stream was set at 20, 40, and 60 ml/min; for spinach, we used 40 ml/min. The stimulus was delivered continuously, or regularly pulsed at 1, 2, 5, 6, 9, or 10 sec half-periods (1 sec pulse = 1 sec odor ON followed by 1 sec odor OFF) over the duration of the experiment.

Experiment Prior to each recording session, each insect was allowed to walk freely on the top of the sphere for 1 min. In order to limit visual cues, the sphere was surrounded by a circle of white paper (30 cm diam, 30 cm high), and the experimental room was illuminated by red light. Each recording session lasted 5 min. We monitored 30 larvae for each stimulus condition and 10 larvae for the blank controls. Each larva was used only once. We used one plant per stimulus condition and airflow frequency i.e., 21 maize plants (3 airflow conditions×7 stimulus conditions) and 7 spinach plants.

Data Measured and Statistical Analyses The raw data (dx_i, dy_i, i=1 to 3000) were first converted into rectangular coordinates ($x_i = \sum_{j=0}^{i} dx_j$ and $y_i = \sum_{j=0}^{i} dy_j$) and averaged down to 1 Hz (X_j and Y_j, j=1 to 300) for further analyses.

Each point of the track at time j was characterized by a displacement vector for 1 sec with the rectangular and polar coordinates (X_j, Y_j) and (L_j, θ_j), respectively. L_j is the distance (in mm) walked during 1 sec, and was computed by $L_j = \sqrt{DX_j^2 + DY_j^2}$. θ_j represents the angle formed by the trajectory vs. the origin, and was defined by $\sin \theta_j = DX_i / L_j$ and $\cos \theta_j = DX_j / L_j$ where $DX_j = X_j - X_{j-1}$ and $DY_i = Y_i - Y_{i-1}$. Walking speed (WS_i, in mm/sec) provided directly L_j. For each track, these values were averaged, and we computed two derived parameters: the "upward length" (UL; in mm) and the straightness (rectilinear index: RI). UL is the summed projection of the displacements made by the insect on the axis parallel to the airflow $UL = \sum_{j=1}^{300} DY_j$. Positive and negative values of UL indicate a track oriented either towards or away from the stimulus. Straightness is computed as $RI = L_{300} / TL$, and varies between 0 (sinuous path) and 1

(straight line). It is the ratio between the length of the track vector (from the origin to the final point of the track, L_{300}) and the length of track: TL, computed as the sum of the length or the elementary displacement vectors $TL = \sum_{j=1}^{300} L_j$.

For each experimental condition (stimulus intensity and frequency), we tested whether the mean angles were distributed uniformly around 360° by Rayleigh *Z* tests. When the uniform distribution was rejected, attraction (angle of mean vector=0°) or avoidance (angle of mean vector = 180°) were tested by using *U* tests as described by Batschelet Batschelet (1981). Calculations and statistical tests were made by using Oriana (Kovach Computing Services, UK).

In addition to the analysis of the angular orientation, we performed an ANOVA by using a GLM procedure followed by multiple mean comparisons with Minitab 12.2 (Minitab Inc., State College, PA, USA). We separately studied walking speed, upward length (UL), and straightness (RI) in order to examine whether the presence of a plant odor and the way it was delivered modified the walking response of the neonate caterpillar. We used a 3-way ANOVA with partial interactions between factors to evaluate the effects of stimulus flow (20, 40, and 60 ml/min), pulse regime (1, 2, 5, 6, 9, and 10 sec pulse, and continuous) and stimulus quality (blank air, maize, or spinach). We could not test the interaction between stimulus quality and stimulus intensity, since we used only one dilution for spinach (40 ml/min). To comply with ANOVA requirements (homoscedasticity for response variables and normal distribution of the residuals), the straightness (RI) was analyzed after an Arcsine(square root) transformation. Although UL values did not follow a Gaussian distribution, the major effects pointed to by the ANOVA are reliable because the method is particularly robust to such divergence in well replicated assays, and clear trends pointed by very high *F* values. Means per experimental groups were compared using the multiple comparison Tukey test at 5% wherever useful (Minitab 12.2). Conclusions drawn from this global analysis were supported fully by non parametrical Kruskal-Wallis tests performed to evaluate the effect of a given factor on UL on appropriate subsets of values, and additional ANOVAs performed on complementary subsets of data with only blank and maize evaluated for all the combinations of stimulation ratio x pulse regime, and with only medium stimulus flow (40 ml / min) with all stimuli: blank, maize, and spinach evaluated over the whole range of pulse regime.

Results

ECB larvae walked continuously and vigorously on the top of the sphere, as soon as they were placed on it. We observed only a few occasional stops during which they lifted their heads up and waved them, as if they were sampling the environment. Under a range of air flow and pulse regimes, the tracks were oriented clearly towards maize odors (Fig. 1a) and directed away from spinach odor (Fig. 1b) while they circled in the presence of simply humid air (Fig. 1c). In order to analyze the tracks, we first examined the mean vector length and angle of the tracks (Table 1). Under this analysis, these two parameters were not found to be significant when larvae were subjected to humidified air (except for pulsing regimes 1 sec and 2 sec at 40 ml/min), when subjected to maize odors delivered at a low airflow (20 ml/min) under several stimulus regimes (1 sec to 6 sec) and at 1 sec stimulus regime for maize at 40 ml/min and spinach at 40 ml/min. The vector length and angle was significant to highly significant with all other stimulus conditions.

We focused our analysis on three variables: walking speed, straightness, and upward length (Fig. 2). We first examined the speed of the larvae under the different stimulus conditions (Fig. 2a and Table 2). The average walking speed across all experimental conditions was 0.67 cm/s (N=1,050). Almost all larvae walked continuously during the experimental session. Speed was highly (P<0.001) affected by all three controlled factors (pulse regime, stimulus flow, and plant odor). This was tested by an ANOVA (Table 2). The average speed observed in the presence of spinach (0.61±0.01 cm/s, N=210; mean±s.e.m.) was significantly lower than with maize (0.70±0.01 cm/s, N=630; P<0.05). Speed in blank (0.65±0.01 cm/s, N=210) was intermediate. The pulse regime strongly (P<0.001) interacted with odor and with the stimulus flow (Table 2).

We then analyzed the straightness (RI) of the tracks (Fig. 2b and Table 2). RI was affected the same way and at the same degree (P<0.001) as speed by the three stimulation parameters. Odors increased the straightness of the paths as compared to the blank ($F_{2.812}$=715). The value of RI under blank air (RI=0.24±0.01, N=210) was significantly lower (about a third) than in the presence of maize (RI=0.75±0.01, N=630) or spinach (RI=0.74±0.01, N=210). Straightness decreased with pulse regime ($F_{6.812}$=6.95) and was affected by the stimulus flow ($F_{2.812}$=56.5). We further tested interactions between these factors. The pulse regime strongly interacted with odor ($F_{12.812}$=7.93) and with the stimulus flow ($F_{12.1049}$=10.1). With maize, the straightness was noticeably lower at 20 ml/min (RI=0.66±0.02, N=210) than at 40 ml/min (RI=0.78±0.01, N=210) or 60 ml/min (RI=0.82±0.01, N=210).

Upward length (UL) was by far the variable that was most influenced by pulse regime and the odor (P<0.001), either directly or in interaction with another parameter of stimulation (Fig. 2c and Table 2.) In turn, a weak direct effect of stimulus flow (P=0.047) was observed. UL was

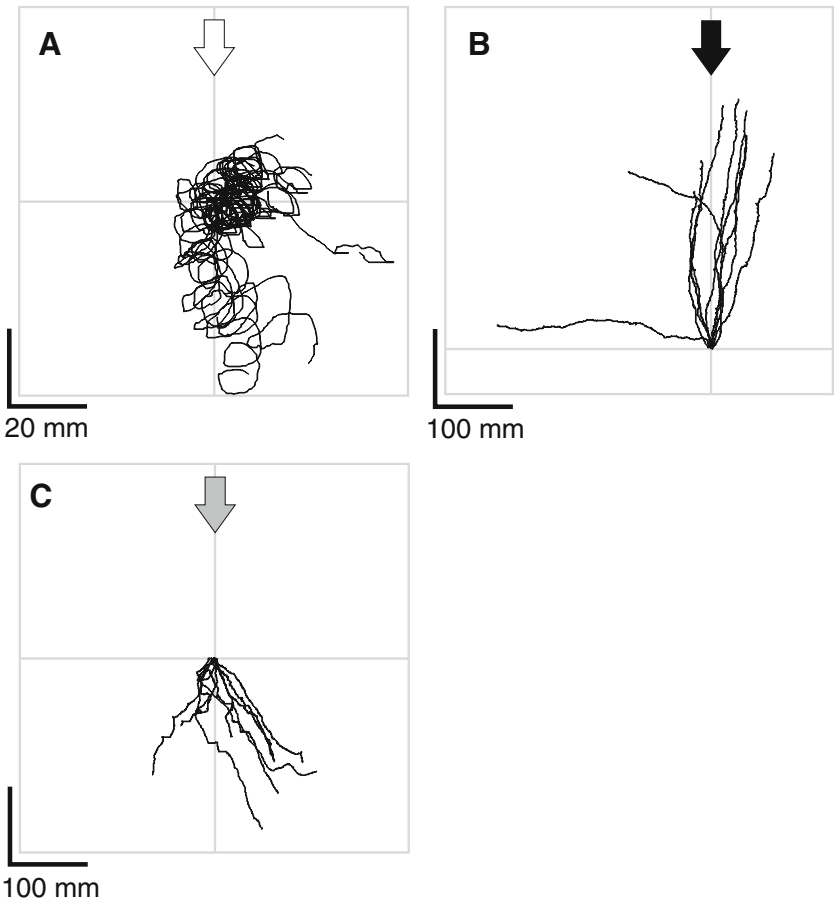

Fig. 1 Representative tracks of neonate ECB larvae walking during 5 min in the presence of different odors on the locomotion compensator. A 80 ml/min stream of air was directed to the top of the sphere (*arrow* on the *top* of each graph) and pulsed at different rates (here: 5 sec stimulus ON followed by 5 sec OFF) and mixed with different ratios of a stimulus flow (here: 40 ml/min) vs. pure air. Each graph displays 10 tracks obtained from 10 different larvae. **a** Odors collected from maize (7 leaves, 80 cm high) enclosed in a nalophane bag. *Horizontal* and *vertical bars* = 100 mm. **b** Odors collected from spinach (17 leaves, 12 cm high). *Horizontal* and *vertical bars* = 100 mm. **c** Humidified air. *Horizontal* and *vertical bars* = 20 mm

dramatically modified by the odor ($F_{2.812}$=134). It was low when no odors were delivered whatever the stimulus flow and pulse regime (UL=6.8±2.5 cm, N=210). This changed in the presence of plant odors. With spinach odor, caterpillars significantly moved downwind (UL=−93± 5 cm, N=210, P<0.05). With maize odors, the average: 14.3±5.6 cm (all stimulus flows and pulse regime N=630) was similar to the odorless situation. However, this value averages contrasting situations, depending on the odor intensity and the pulsing rate. ECB caterpillars moved opposite to the stimulus when maize odor was delivered continuously (UL=−178±10 cm at 60 ml/min; UL=−122± 14 cm at 20 ml/min). Long pulses (9 and 10 sec) also were repellent, except at 20 ml/min where pulsing with 10 sec stimuli sufficed to restore an attraction (UL=120±10). The direction of the tracks clearly depended on stimulus flow and pulse regime.

Discussion

ECB neonate larvae orient towards or away from maize odors depending on the air flow and on the pulsing rate. They orient away from spinach odors at the air flow of 40 ml/min irrespective of the stimulus structure above 1 sec pulses. In the absence of plant odors, ECB larvae tend to walk in a circle and do not express any anemotactism. However, they are able to use the wind direction, when they have experienced odors, to maintain their direction during time intervals of up to 10 sec. Since the larvae tested here were never exposed to a plant since hatching, these experiments reflect their innate orientation strategies in response to plant odors. Spinach odors were repellent within the air streams tested, suggesting that they contain compounds that contribute to plant defense, in addition to the production of phytoecdysteroids. Maize odors exerted either an attraction or repellency depending on the stimulus conditions, e.g., air stream and pulse regime.

These results confirm and extend previous studies on the orientation of neonate larvae that express a range of locomotory and orientation responses towards host plant odors (Harris et al. 1999; Müller and Hilker 2000; Singh and Mullick 2002; Castrejon et al. 2006) or towards plants damaged by conspecifics (Singh and Mullick 2002; Carroll et al. 2008). However, nearly all of these studies used different olfactometers ranging from simple olfactory tubes to “artificial plants”. Such diversity makes difficult any comparison between insect species. The great advantage of

Table 1 Orientation of neonate ECB larvae under various odor stimulation and pulsing regimes: lengths and angles of the mean vectors of the recorded walking tracks

	Stimulus	Mean vector	Pulse regime						
	Flow	Properties	1 sec [a]	2 sec	5 sec	6 sec	9 sec	10 sec	Cont.
Blank	20 ml/min	Length (R) [b]	0.22	0.20	*0.41*	*0.21*	*0.78*	*0.57*	*0.19*
		Angle (°) [c]	156±100	21±103	*17±77*	*194±102*	*3±41*	*−6±61*	*172±104*
	40 ml/min	Length	**0.58 ***	**0.59 ***	*0.40*	*0.24*	*0.15*	*0.20*	*0.20*
		Angle (°)	**6±60 (+)****	**9±58 (+)****	*180±78*	*143±96*	*83±111*	*185±103*	*19±103*
	60 ml/min	Length	*0.41*	*0.41*	*0.41*	*0.38*	*0.21*	*0.40*	*0.14*
		Angle (°)	*19±77*	*25±76*	*173±77*	*182±79*	*5±101*	*4±77*	*90±114*
Maize	20 ml/min40 ml/min	Length	*0.13*	*0.21*	*0.25*	*0.23*	**0.47**	**0.86**	**0.73**
		Angle (°)	*89±116*	*232±101*	*141±95*	*115±98*	**20±70 (+)**	**9±31 (+)**	**167±46 (−)**
		Length	0.23	**0.94**	**0.73**	**0.89**	**0.67**	**0.68**	**0.90**
		Angle (°)	80±100	**2±21 (+)**	**−9±45 (+)**	**14±28 (+)**	**181±51 (−)**	**169±50 (−)**	**180±26 (−)**
	60 ml/min	Length	**0.90**	**0.91**	**0.95**	*0.29*	**0.78**	**0.46**	**0.95**
		Angle (°)	**4±27 (+)**	**−2±25 (+)**	**14±18 (+)**	*39±90*	**164±40 (−)**	**174±71 (−)**	**182±19 (−)**
Spinach	40 ml/min	Length	*0.24*	**0.84**	**0.89**	**0.88**	**0.91**	**0.95**	**0.91**
		Angle (°)	*49±97*	**182±34 (−)**	**171±28 (−)**	**173±29 (−)**	**167±26 (−)**	**185±18 (−)**	**177±25 (−)**

[a] Pulse regime: 1, 2, 5, 6, 9, 10 sec. (1 sec = 1 sec odor ON followed by 1 sec odor OFF); cont. = continuous stimulation.

Average orientation is given by the mean vector of the tracks, defined by its length R and the angle to the wind direction. R varies between 0 and 1 and its variance equals 1-R: the straighter and the most similar the directions of the tracks the closer R to 1. Bolds values indicate that random orientation can be rejected with $P<0.001$, or $P<0.01$ (**) or $P<0.05$ (*)

[b] Bold R indicate that the directions of the elementary vectors of the tracks are significantly distributed around the value of the angle of the mean vector (Rayleigh tests).

[c] Bold values (mean±s.e.m.) indicate that the angle of the mean vector does not differ from 0° (+), upwind walk, or 180° (−), downwind walk (V tests).

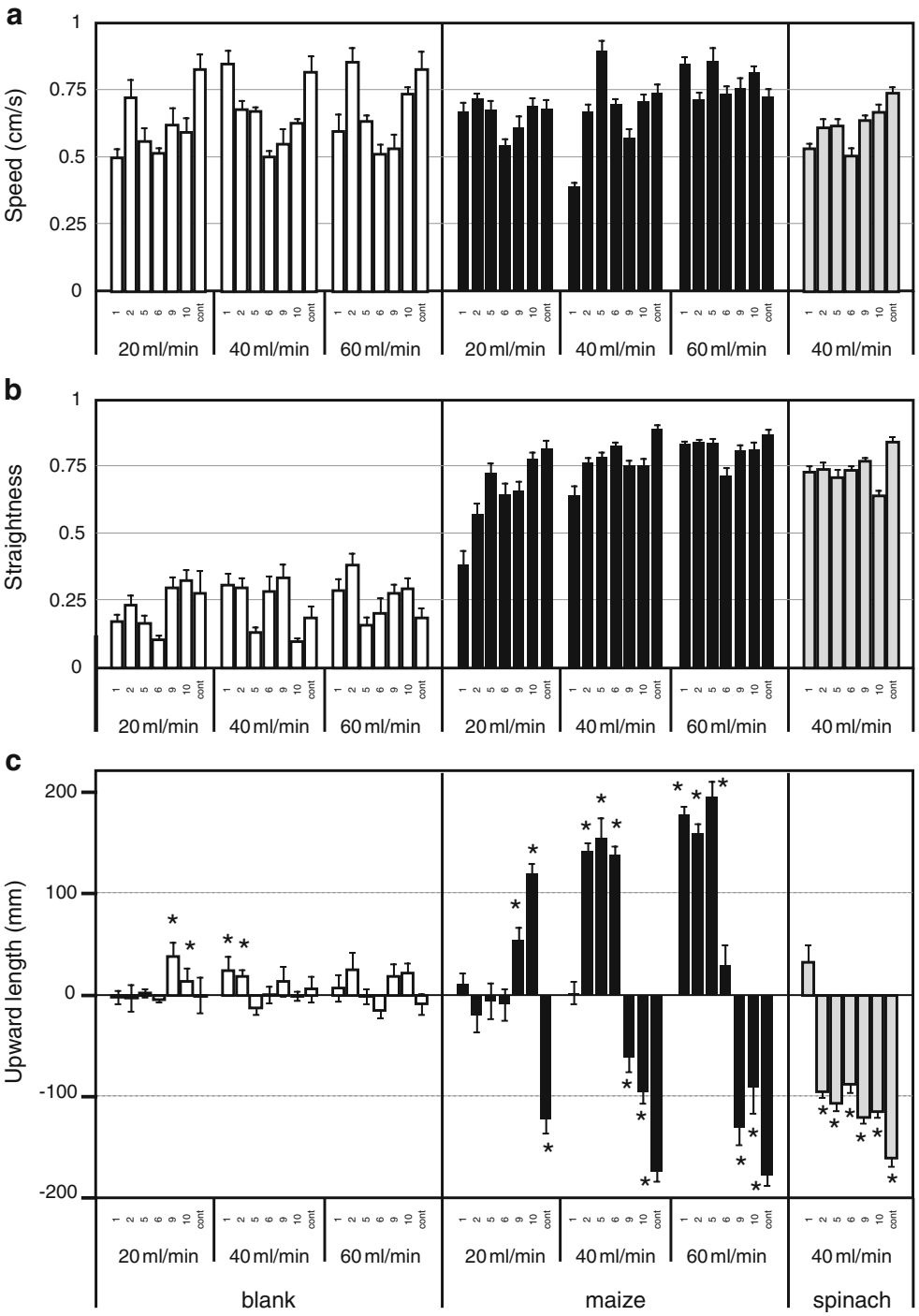

Fig. 2 Histogram of the parameters (mean ± s.e.m.) measured from tracks recorded with different odors and stimulus regimes. Three odor conditions were tested: humidified air (*white bars*; N=10 larvae per stimulus condition), maize (*black bars*) and spinach (*grey bars*) volatiles (N=30 larvae per stimulus condition). These odors were delivered at different airflows (20, 40, 60 ml/min) and under different pulse regimes (1, 2, 5, 6, 9, 10 sec pulses and continuous odor). See text for the significance of the parameters displayed. **a** Speed (cm/s). **b** Straigthness. **c** Upward length (mm): * indicate when the mean vector was significantly oriented up- or downwind

a locomotion compensator over other approaches is that it allows exploring odorant-mediated anemotaxis in an open field without influence from other stimuli (surface structure, gravity, limits of the arena, etc) and allows recording trajectories and decomposing them easily into different components.

ECB neonate larvae express a robust walking behavior on a locomotion compensator. Even in the absence of any stimulus, they move continuously, provided they are hungry. Once ECB larvae experienced an odor and chose an orientation, they usually kept walking in the same direction even when the odor was removed from the airflow for several seconds (up to 10 sec). This strategy seems adapted to cope with erratic messages provided by odor plumes in a turbulent environment. Actually, adult insects generally orient better in the presence of intermittent stimuli than within a continuous odor plume when they are flying (Baker et al. 1985; Cardé and Willis 2008). For walking

Table 2 Effect of stimulation conditions on the displacement of ECB larvae: excerpt of the ANOVA tables showing F values for the sources of variation evaluated

Source of variation (df)	Pulse regime (PR) (6,812)	Stimulus flow (SR) (2,812)	Odor (2,812)	PR x SR (12,812)	PR x odor (12,812)
Walking speed	14.2	29.6	9.34	5.88	6.70
Straightness (RI)	6.95	56.5	715	10.1	7.93
Upward length (UL)	31.6	3.07 *	134	46.1	33.4

All factors and binary interactions significantly contributed to the displacement variables ($P<0.001$ for F values) with the exception of the stimulus flow whose contribution to Upward length was much weaker (* $P=0.047$).

insects, intermitted stimuli improve orientation in carrion beetles (Böhm 1995) and Colorado potato beetle larvae (Hammock et al. 2007) but have no effect in others, such as haematophagous bugs that orient towards pulsed CO_2 (Barrozo and Lazzari 2006) or possibly *Lobesia botrana* larvae, which stop and search for odor cues upon cessation of the stimulus (Becher and Guerin 2009).

While flying insects may need to adopt an active searching strategy to cope with the erratic nature of odorant stimuli in the open air, larvae that are already on their host plants or on their food (Gerber and Stocker 2007) remain confined to the boundary layer of their plant, and may not need to care about the temporal structure of the stimulus. Actually, ECB larvae do not seem to improve their orientation with pulsed stimuli as compared to Colorado potato beetle larvae, which better orient towards intermittent stimuli (1:1 sec pulses) than to a continuous odor (Hammock et al. 2007). Our observations merely show that they are capable of taking into account discrete odor stimuli over a large range of pulsing rates and of modulating their search strategy accordingly. The most striking feature of these observations is that once larvae have been stimulated by a pulse of odor, they lock to the direction chosen even when the stimulus is interrupted by 10 sec. Further studies are needed to elucidate how long this memory is maintained, and if neonate larvae are able to display casting as observed in adults (Cardé and Willis 2008).

The reversal of orientation of ECB larvae stimulated with maize odors at different pulse regimes was unexpected, and it is not clear why it happened. Short odor pulses of 1 sec up to 6 sec induced an attraction when odors were delivered at 40 and 60 ml/min. Odor pulses of 9 sec or longer were repellent. At the lowest stimulus flow (20 ml/min), there was no significant orientation towards short odor pulses, while 9 and 10 sec pulses induced a clear attraction, and continuous stimulation was repellent. One could argue that this effect is due to differences among plants (because single plants were used for each pulse regime in our experimental protocol). This hypothesis can be ruled out because we would then expect a random effect of the pulse regime, while we observed sequential increases or decreases in upward length according to the pulse regime.

Several hypotheses could account for the effect of the pulsing regime on the attractivity or repellency of maize odors. First, the odor mixture might change in intensity and in quality with the stimulus regime. In our experimental setup, the air was pushed through the bag containing the plant on demand. Longer stimulus pulses might exhaust the bag's content and induce the release of different quantities of odorants than when discrete amounts of air are sampled with short stimulus pulses. These odorants can be either repellent or less attractive to ECB larvae, depending on their concentration in the air stream. For example, in *Drosophila* (and other insects), odorants are attractive or repellent depending on the concentration (Acebes and Ferrus 2001; Devaud 2003), and this effect is linked both to the nature of the odor and to the number of glomeruli activated during the response (Semmelhack and Wang 2009). Second, olfactory receptors of ECB larvae may adapt to some odorants when delivered continuously, as in *Drosophila* larvae (Cobb and Domain 2000), but not when these odors are pulsed. This issue could be resolved by detailed electrophysiological studies. Third, larvae could integrate the temporal structure of the odorant stimulation in their search strategy. If the larvae are not on a plant, odors are likely to be intermittent; or the opposite, if larvae are on a plant, odors are likely to be continuous and concentrated. However, one would expect such odors to induce an arrestment and a local search, rather than a reversal of the direction of orientation. Further studies are needed to clarify these hypotheses, for example, by re-designing the odor delivery system (using a continuous flow over the plant), by sampling and analyzing odors released in different pulsing conditions, by correlating more precisely the timing of the odor stimulation with the behavior of the larvae (as in Hammock et al. 2007), or by

using synthetic stimuli allowing a better control over the concentrations released.

In this work, we tested the hypothesis that ECB larvae would walk away from spinach, which is unsuitable as a host plant. Spinach odors were repellent over the whole range of pulsing rates used in this experiment except with 1 sec pulses that elicited no orientation. This is reminiscent of 1 sec maize odor pulses delivered at 20 and 40 ml/min that elicited no orientation, possibly because of a low odor concentration. It remains to be tested whether spinach odors delivered at a different concentration are still repellent or if they could become stimulatory. From a neurobiological point of view, some odors induce either attraction or avoidance depending on the concentration, while others induce aversive reactions (Kreher et al. 2008; Semmelhack and Wang 2009). Our present data cannot resolve the issue, which should be approached by testing different odors dilutions of spinach, using the same approach as with maize.

That spinach odor might be repellent to ECB larvae is particularly interesting. From previous work, we know that spinach contains phytoecdysteroids, primarily 20-hydroxyecdysone (Schmelz et al. 1998). Phytoecdysteroids are composed of a sterol ring with a lateral chain and are non-volatile molecules found in plants and other organisms (Lafont 1997). Most of them are agonists or antagonists of insect molting hormones, thus making them good candidates as natural pesticides (Marion-Poll et al. 2005). Our observations suggest that ECB larvae detect specific volatiles emitted by spinach, which are either directly linked to the metabolism of this family of molecules or produced in addition to them, or which correspond to a combination of green volatiles emitted at different flows than maize.

Finally, one wonders how spinach odors or maize odors would affect the orientation behavior of larvae when they are emitted within an odorant background. Earlier experiments on *Leptinotarsa decemlineata* using a locomotion compensator, showed that potato odors could be either masked by the odorant background (Thiery and Visser 1986) or mislead by the odor of a non-host plant (Thiery and Visser 1987). This could be of importance in assessing the impact of mixed cropping not only on female oviposition but also on larval dispersal within fields.

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either parent); an additive pattern, whereby hybrid traits are intermediate between the two parental types; or no difference between hybrids and parental taxa (Fritz et al. 1999). None of these responses dominates in studies published to date. In 127 studies of susceptibility of plant species and their hybrids to attack by herbivorous insects, hybrid susceptibility appears the most common pattern (39%), and hybrid resistance appears to be reasonably rare (10%), while 22% of studies identified an additive pattern, and almost one-third (29%) found no differences between parents and hybrids (Fritz et al. 1999; Dungey and Potts 2003; Hallgren et al. 2003; O'Reilly-Wapstra et al. 2005). An additive pattern has been found in interactions between eucalypt hybrids and mammalian herbivores (Scott et al. 2002).

Development of interspecific eucalypt hybrids for forest plantations is a silvicultural strategy adopted in many eucalypt-growing regions worldwide to maximize tree performance by combining the desirable traits of different species (de Assis 2000). Eucalypts are known to form hybrids readily with related species (Griffin et al. 1988). Traits for improvement through hybridization include growth rate, coppicing and propagation ability, pulp yield, wood density, and resistance to frost, drought, salinity (Dale and Dieters 2007), and pests and diseases (Potts and Dungey 2004). The eucalypts are a diverse group of trees and shrubs (> 800 species), generally considered to belong to the genera *Eucalyptus* (Brooker 2000), *Angophora,* and *Corymbia* (Ladiges and Udovicic 2000; Ochieng et al. 2007a, b) in the family Myrtaceae. One prominent characteristic of the group is the high essential oil content of the leaves, and the oils vary substantially among taxa (Bignell et al. 1998; Dunlop et al. 1999; Asante et al. 2001; Keszei et al. 2008), thus affecting feeding preferences of insect herbivores (Edwards et al. 1993; Steinbauer et al. 2004). An understanding of how characters important to plant herbivores (e.g., secondary chemicals and physical leaf characteristics) vary between species and their hybrids enables an understanding of the mechanisms of host choice by insect herbivores (Hallgren et al. 2003).

We examined variations in foliar chemical composition, leaf physical characteristics, and feeding by herbivorous beetles with three taxa: two allopatric species (*Corymbia citriodora variegata* (CCV) and *C. torelliana* (CT)) and their hybrid (CT × CCV). All are important in subtropical hardwood plantation forestry where the hybrids have significant advantages in growth, and tolerance to disease, insects, and frost, and also have been successfully vegetatively propagated (Lee 2007; Lee et al. in press). This tolerance to insects is, however, anecdotal, and needs quantification. The model pest species chosen to examine patterns of herbivory was *Paropsis atomaria* Olivier (Coleoptera: Chrysomelidae: Paropsina), a major pest of the commercially valuable CCV (Carnegie et al. 2008). *Paropsis atomaria* is an ideal model for this study, as adults and larvae feed on the same foliage, and thus allow a test of linkages between oviposition preference and larval performance. In addition, the test taxa represented a known host (CCV), a novel host (CT), and their F1 hybrid (CT × CCV).

Methods and Materials

Study System

Corymbia citriodora subsp. *variegata* (CCV) belongs to the Section Politaria, and recent studies have shown that it is genetically indistinguishable from *C. citriodora* subsp. *citriodora* and *C. henryi* (Ochieng et al. 2008; Shepherd et al. 2008), although the taxa are chemically distinct (Asante et al. 2001). It has a sympatric distribution with *P. atomaria*, although it was recorded only recently as a host (Nahrung 2006). *Corymbia torelliana* (CT) occurs naturally in about a 350×80 km zone in northern Queensland (Boland et al. 1992), a vicinity to which *P. atomaria* has recently expanded its range (Nahrung 2006). *Corymbia torelliana* (CT) is not a host for *P. atomaria*, although around 20 other eucalypt species are (CAB International 2005). An artificial hybrid between CT and CCV has been prepared for commercial purposes, and is planted widely throughout the insect's range (Lee 2007). All lifestages (except pupae) of *P. atomaria* occur on the host plant, with oviposition by females determining subsequent larval feeding habitat, and the long-lived adults and all four larval instars feeding on new growth, removing apical leaves. This results in a characteristic broom-topped appearance to trees (Carne 1966).

Foliage Collection

Seed was collected from one open-pollinated tree of each pure taxon (i.e., CT and CCV), thus ensuring that all samples had the same mother (were at least half-sibs). Hybrid (CT × CCV), seed was collected from one CT mother artificially pollinated from a single CCV father (i.e., full sibs). Neither of the hybrid parents were the same trees (families) as the pure taxon, so as to ensure a more representative sampling rather than looking only at intra-familial responses. Plants were sown from seed in potting mix comprising 50% pine bark fines, 25% peat (Aussie Peat) composted, and 25% perlite to which Osmocote® and Ag lime were added each at 4 kg/m^3, and gypsum, Micromax (fertilizer) and Hydroflow (wetting agent) were added each at 1 kg/m^3. Seedlings were raised in the glasshouse for the first 6 wk under mist, and then put under shadecloth for 2 wk before being put out into full

sun. Plants were repotted later into 130 mm diam pots and housed in a glasshouse (24°C, ambient light) for several months prior to use in experiments.

Foliage used in all experiments (physical analysis, chemical analysis, and feeding trials) was sourced from about 20 individual plants of each taxon. Only the first two—four fully expanded apical leaves were used for all trials to standardize the age of foliage during testing.

Foliar Analyses—Physical Analysis

Moisture content and specific leaf weight (SLW) (used as an indicator of leaf toughness—see Steinbauer 2001) were determined by measuring the fresh weight (FW) of leaves (to nearest 0.001 g), drying them in paper envelopes at ambient temperature for 3 wk, and re-weighing them (DW). Leaf area (mm^2) was estimated by using Compu Eye Leaf and Symptom Area software (Bakr 2005). Moisture content was calculated as (FW - DW)/FW, while SLW was determined as DW/area.

Foliar glabrousness was determined as the mean number of leaf trichomes in the field of view of a dissecting microscope (×40). The thickness (width) of the leaf lamina was measured under a dissecting microscope (×40) by cutting 4 small strips (~5 mm wide) and averaging the measurements per leaf (N=12). Data were analyzed using StatView (V 5.0.1). One-way *ANOVA* was used to analyze moisture content (following arcsine-square root transformation), SLW, and lamina thickness, with *Fishers LSD* test used for *post-hoc* comparisons. *Kruskall Wallis* test was used for leaf glabrousness, as data were not normally distributed. Twelve replicates of each foliage type were conducted for each parameter.

Foliar Analyses—Chemical Analysis

Replicate samples (N=5) of foliage (2.07±0.019 g FW) from 5 randomly chosen plants of each taxon were collected, and cut into squares (≤ 1 cm^2), and extracted with hexane (Sigma-Aldrich ≥ 99%) (≈ 15 ml) for 50 min, stirring for 1 min, three times within this period. The extract was filtered through filter paper (Whatman) and stored in the freezer (−20°C) until analysis (Jones et al. 2002; Rapley et al. 2004c).

Samples (1 µl) were analyzed with a gas chromatograph (GC) (Agilent 6890 Series) coupled to a mass spectrometer (MS) (Agilent 5975) and fitted with a silica capillary column (Agilent, model HP5-MS, 30 m×250 µm ID × 0.25 µm film thickness). Data were acquired under the following GC conditions—inlet temperature: 250°C, carrier gas: helium at 51 $cm.s^{-1}$, split ratio 13:1, transfer-line temperature: 280°C, initial temperature: 40°C, initial time: 2 min, rate: 10°$C.min^{-1}$, final temperature: 260°C, final time: 6 min. The MS was held at 280°C in the ion source with a scan rate of 4.45 $scans.s^{-1}$.

Peaks that were present in blank hexane (control) samples were discarded from analysis in test samples. Tentative identities were assigned to peaks with respect to a Kovats Retention Index analysis and the National Institute of Standards and Technology (NIST) mass spectral library. Mass spectra of peaks from different samples with the same retention time were compared to ensure that the compounds were indeed the same.

The presence of peaks in the chromatograms, and their relative areas were analyzed by nonparametric methods (*Bray-Curtis* cluster analysis and multidimensional scaling (*MDS*) ordination) (Clarke 1993) to ascertain whether any differences could be detected among the samples. The use of relative percentage area for the peaks removes the need for standardizing concentrations from samples where slightly different total mass of components has been extracted from leaf material. Instead, it is the relative amount of each component that is compared, thus ensuring that comparisons can be made among samples of unknown total concentrations.. Each point in the *MDS* plot represents an individual plant, and points that are close together (clumped) correspond to individuals with similar peak composition (presence and abundance). Since they represent relative differences among samples, the axes of an *MDS* plot are dimensionless. *MDS* has been used successfully in previous studies to analyze chromatographic data (e.g., Hayes et al. 2006).

To determine whether clusters of individual plants relating to the taxa investigated were significantly different from each other, we used an analysis of similarity (*ANOSIM*). The *ANOSIM* tests are a range of Mantel-type permutations of randomization procedures, which make no distributional assumptions. These tests depend only upon rank similarities, and thus are appropriate for this type of data. We used a similarity percentages (*SIMPER*) analysis to determine which peaks were the most important in contributing to any differences between groups, and to assess similarity between individuals within each group. The software used for the multivariate analysis was Primer 5 for Windows (V 5.2.9, Clarke and Gorley 2001).

Herbivory Trials—Field Assessment

Two field sites in Queensland (Site I S 26.595° E 151.915°; Site II S 26.101° E 151.623°) containing CT, CCV and CT × CCV were monitored for the incidence (proportion of trees with damage) of characteristic damage caused by *Paropsis atomaria*. Both plantations were established in March 2004 so were the same age at time of sampling (January 2008). Site I comprised a single tree plot design with each family/seedlot represented by one randomly

allocated individual in each of twenty blocks of 100 trees each. One half (i.e., 10 blocks) of the entire site was sampled. Site II comprised 20 lines of 10 trees each. Each plant type was represented by at least 4 such line-plots, each representing a different family/seedlot except CCV which was represented by only one line-plot. Larvae, beetles, and egg batches were present at both sites when censuses were conducted. Each tree was scored for its suitability as a host for *P. atomaria* by recording the presence or absence of *P. atomaria* lifestages. χ^2-pairwise comparisons were made for each site to compare the beetle incidence on each taxon.

Herbivory Trials—Laboratory Bioassays

The same plants used in the chemical and physical foliar attribute studies were used to provide foliage for laboratory bioassays with *P. atomaria*.

Herbivory Trials—Larval (No-Choice) Trials

Field-collected egg batches were held in a controlled-temperature cabinet at 25°C, 16L:8D photoperiod. Unfed (except on egg chorion) neonate larvae were transferred with a soft paintbrush, with larvae from different egg batches divided among treatments to control for possible maternal effects. Eight larvae were placed directly onto test foliage for each replicate (N=11 per taxon). The experiment was conducted in a controlled temperature cabinet at 16°C, 16L:8D. The group size was selected as that above which mortality was constant (Duffy et al. 2008), while 16°C represented the temperature at which mortality was lowest (Nahrung et al. 2008) in previous laboratory studies. A piece of moist filter paper was provided to slow desiccation of treatment foliage. Mortality was recorded, old foliage and filter paper were removed, and fresh filter paper and foliage were added every 3–4 days. Care was taken to ensure that larvae were provided an excess of foliage, such that they never consumed all foliage present. When larvae reached the fourth instar, the replicate was transferred to a larger plastic cage (160×110×35 mm), and upon pre-pupation (dorso-ventral flattening and cessation of feeding) individuals were transferred to separate, numbered, sterilized-soil-filled cells of a plastic modular tray (cell dimensions 20×20 mm). When pupae formed, they were weighed (to nearest 0.001 g) on an electronic balance, and returned to their cell until adult emergence. On emergence, sex was determined under a dissecting microscope (×40), using tarsal differences of the foreleg as the discriminating factor (Baly 1862).

Overall larval mortality, development time and pupal weight were used to assess larval performance on the different foliage types. One-way *ANOVA* was used to compare foliage type among these performance parameters, with proportion data arcsine-square root transformed prior to analysis, and *Fisher's LSD* test used to *post-hoc* test. A *Kaplan-Meier* survival curve (Kaplan and Meier 1958) was plotted for each foliage type, and non-parametric pairwise comparisons were made (P<0.05) to compare larval survival rate on different taxa.

Herbivory Trials—Adult Feeding (Choice) Trials

Field collected beetles were housed in gauze cages and provided with fresh *Eucalyptus cloeziana* foliage prior to use. Twenty-four hours before the start of the trial, beetles were removed from foliage, their sex determined as above, and deprived of foliage until the trial began. For each replicate, one male-female beetle pair was placed into a plastic arena (160×110×70 mm). An apical branch comprising the first 2–4 fully expanded leaves of each foliage type was inserted through holes in the base of the cage into water below. Visually-estimated equivalent biomass was provided of each type in each replicate; twelve replicates were run simultaneously under ambient laboratory conditions. The experiment ran for 3 d, after which adults were removed and the remaining area of each leaf was estimated by placing it under a clear plastic sheet of grid-squares (3×3 mm) and counting the number of squares (to nearest 0.25 of a square) of foliage. The amount of foliage eaten was determined by multiplying the number of grid-squares by 9 mm^2. One-way *ANOVA* was used to detect differences between treatments, and a *Fisher's LSD* test was used to identify where those differences lay. Data for all herbivory trials were analyzed with StatView (V 5.0.1).

Results

Foliar Analyses

Moisture content was the only parameter measured not to differ significantly among different taxa (Table 1). *C. ciriodora* subsp. *variegata* (CCV) had the highest specific leaf weight and lamina thickness, and no leaf hairs (Table 1). In almost all cases, results for the hybrid either lay between that of each parent, or was not different from CT.

The compounds identified in extracts were primarily mono- and sesquiterpenes, as well as some waxes and long-chain hydrocarbons that could not be identified unambiguously. The percentage of individuals in each taxon group from which the component was identified is shown (Appendix 1). Chromatograms produced from hexane extracts of leaves of the three taxa were distinctly different from each other. The composition of components was consistent between replicates / individuals, however, the relative amounts varied among

Table 1 Moisture content, SLW, lamina thickness and glabrousness of *Corymbia citriodora* subsp. *variegata* (CCV), *Corymbia torelliana* (CT) and their hybrid (CT × CCV). The final row shows analysis results, and different letters within columns designate significant differences between taxa

Taxon	Moisture content (%)	Specific Leaf Weight (mg/mm^2)	Lamina thickness (μm)	Leaf surface glabrousness (# trichomes)
CCV	77.2±1.6	0.098±0.007 a	126.2±5.4a	0a
CT × CCV	74.0±1.9	0.052±0.006 b	102.4±4.3b	87.3±25.3b
CT	80.0±0.8	0.054±0.007 b	88.7±3.9c	106.9±23.6b
ANOVA/Kruskall-Wallis results	$F_{2,30}$ = 2.3 P=0.12	$F_{2,30}$ = 13.9 P<0.001	$F_{2,33}$ = 17.4 P<0.001	H_2 = 23.79 P=0.03

taxa. The CT samples were highest for the late-eluting components, the CCV samples highest for the early-eluting components, and the CT × CCV samples either intermediate between the two parental species or showing an additive response (Fig. 1).

In addition to visual chromatographic differences, the samples were statistically distinguishable, and pairwise comparisons demonstrated that all taxa differed from each other (*ANOSIM*: *Global* R=0.814, P=0.001; CCV, CT: R=0.964, P=0.008; CCV, CT × CCV: R=0.834, P=0.008; CT, CT × CCV: R=1, P=0.008). The *MDS* output (Fig. 2), provides a visual representation of the data described by the *ANOSIM*. Each point on the figure represents an individual extract. Points that are close together are more similar, and those farther away are more different.

The *SIMPER* analysis is a measure of the similarities of samples within a defined grouping (in this case taxa studied). All groups have high levels of similarity, but the CCV samples are the most dispersed (Fig. 2).

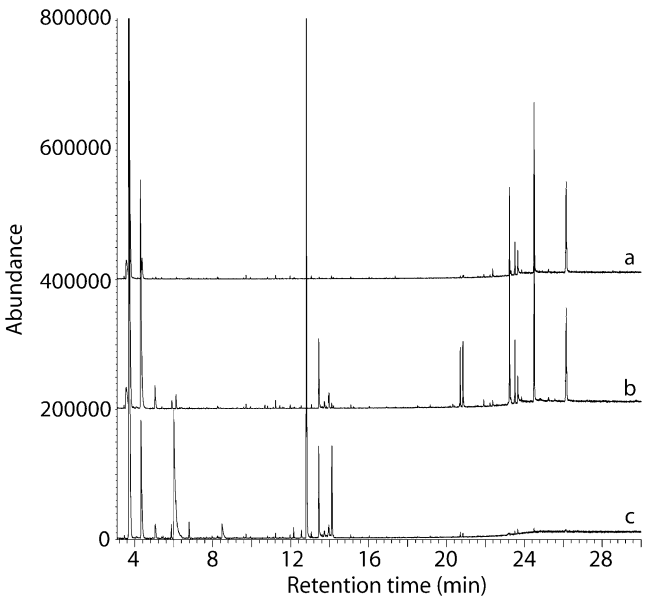

Fig. 1 Chromatograms produced by hexane extracts of *Corymbia* leaves. A typical extract from *Corymbia torelliana* (CT) is shown on top (**a**), a typical extract from *Corymbia citriodora* subsp. *variegata* (CCV) is shown on the bottom (**c**), and a typical extract from the hybrid (CT × CCV) is shown in the center (**b**)

The mean percentage area (± s.e.) under the peak for the most important peaks used to distinguish between the taxa are listed (Appendix 2). These peaks account for over 50% of the total dissimilarity between the groups. The peaks are listed in the table in order of increasing dissimilarity between the groups, i.e., the first peak contributes most to the overall dissimilarity. Retention time is as given in Appendix 1.

Herbivory Trials

The proportion of trees associated with *P. atomaria* did not differ between sites (χ^2 = 3.04, P=0.08) so data from the two sites were combined for subsequent taxa-level analysis. Three-quarters (153/204) of CCV trees were damaged by *P. atomaria*, while less than 30% (67/232) of CT trees exhibited damage symptoms. Sixty-five percent (202/313) of hybrid CT × CCV trees were associated with *P. atomaria.* Again, the hybrid was intermediate between the parental taxa. Pairwise comparisons (Bonferroni-adjusted, P=0.02) demonstrated that each taxon differed significantly from the others (CCV, CT χ^2 = 92.4, P<0.001; CCV, CT × CCV χ^2=6.3, P=0.01; CT, CT × CCV χ^2 = 67.8, P<0.001).

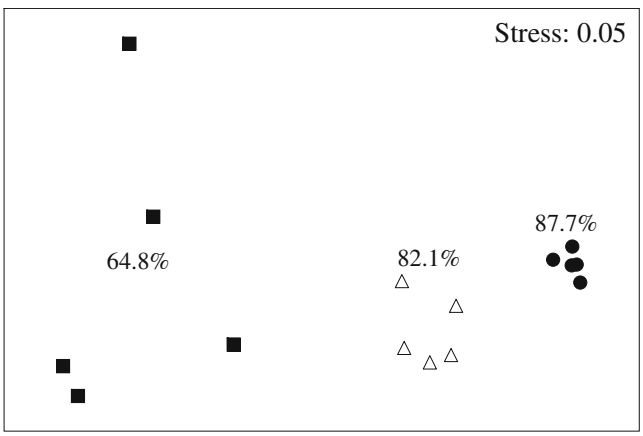

Fig. 2 Two-dimensional MDS ordination of the 15 *Corymbia* extracts including SIMPER measures of average similarity. The plot is based on fourth-root transformed abundances and a Bray-Curtis similarity matrix. Extracts from each taxon cluster separately. Symbols: *Corymbia citriodora* subsp. *variegata*-CCV (■), *Corymbia torelliana*-CT (●), their hybrid-CT × CCV(△)

Table 2 Mean ± s.e. mortality, development time and pupal weights of *Paropsis atomaria* larvae reared on *Corymbia* taxa. Different letters within columns designate significant differences between taxa

Taxon	Larval mortality (%)	Development time (days)	Pupal weight (g)	
			male	female
CCV	84.1±6.8 a	42.6±1.0	0.12±0.01 a	0.18±0.01 a
CT × CCV	54.5±8.3 b	42.6±0.9	0.10±0.01 b	0.14±0.01 b
CT	51.1±6.4 b	40.9±0.5	0.12±0.01 a	0.17±0.01 a

Overall larval mortality (proportion dying before pupation) was highest on CCV (Table 2) (*ANOVA*: $F_{2,30}$ = 8.5, P=0.001), although mortality *rate* differed significantly only between the two parent taxa (Fig. 3) (test statistic = 5.1, P=0.02). Larval development time did not differ according to rearing host (*ANOVA*: $F_{2,21}$ = 1.5, P=0.25), but pupal weight differed depending on sex (males were smaller than females) and natal host taxon (*ANOVA*: sex: $F_{1,36}$ = 70.0, P<0.001; taxa: $F_{2,36}$ = 9.2, P=0.0006, sex*taxa: $F_{2,36}$ = 2.2, P=0.13; Table 2), with hybrids resulting in significantly smaller adults than parent taxa for both sexes.

Feeding occurred on CCV in all replicates, whereas only 40% of replicates of CT and CT × CCV sustained adult feeding damage. The amount (mm^2) of foliage eaten differed among taxa (*ANOVA*: $F_{2,27}$ = 17.12, P<0.001), with CCV the most-preferred host (Fig. 4).

Discussion

Although there is some variability in the response, the hybrid exhibited traits intermediate to the parent species for several of the foliar characteristics investigated, and the feeding preference of *P. atomaria* followed a similar trend in both the laboratory and field (Table 3). The known host, CCV, recorded the highest field incidence of *P. atomaria*, and laboratory trials supported this pattern, with CCV the most-preferred taxon in adult feeding trials. Almost one-fifth of the foliar chemical components identified were detected only in CCV, and it may be one (or many) of these compounds that are involved in host location and selection for this species. The one with the greatest concentration was elemol, a sesquiterpenoid that has been reported previously as a dominant component of CCV leaf chemistry (Asante et al. 2001). Similarly, limonene was present in CCV and the hybrid, but was not detectable in CT. Limonene is a well known attractant for a range of insects, especially beetles (Chenier and Philogene 1989; Miller 2007). The monoterpene α-pinene is a common component of eucalypt leaf chemistry (Asante et al. 2001; Bignell et al. 1998), that varies among the taxa in this study, with high levels in both the host plant and the hybrid, and low levels in CT. α-Pinene is a known kairomone for Colorado potato beetle (*Leptinotarsa decemlineata,* Coleoptera: Chrysomelidae), and attracts the beetle (Panasiuk 1984). The high levels of both limonene and α-pinene in the host taxon and hybrid, and its lack of detectibility in extracts of the non-host plant, may explain some of the variation in behavior observed in our study.

Only two compounds were detectable in the non-host parent (CT) alone, and it is possible that these are repellent to *P. atomaria*. Ohmart (1991) speculated that adult paropsine beetles were repelled or unattracted to the volatile

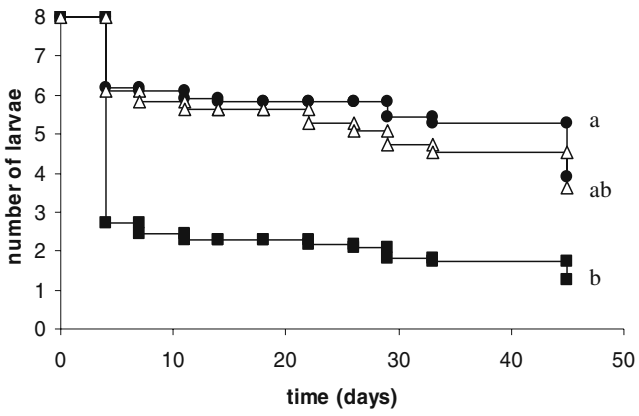

Fig. 3 Kaplan-Meier survival curve illustrating the mortality rate of *Paropsis atomaria* larvae reared on *Corymbia citriodora* subsp *variegata*-CCV (■), *Corymbia torelliana*-CT (●) or their hybrid-CT × CCV (Δ)

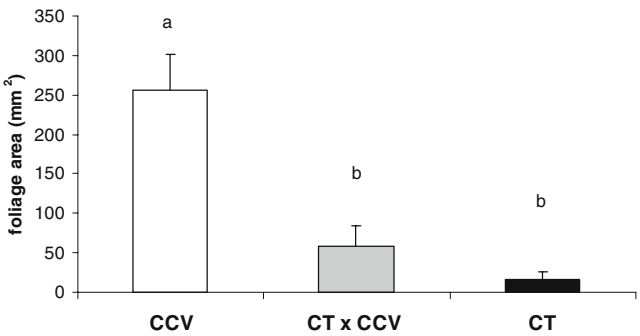

Fig. 4 Mean + s.e. amount of *Corymbia* taxa foliage consumed (mm^2) by one male-female pair of *Paropsis atomaria* adults

Table 3 Summary of foliar attributes and responses of *Paropsis atomaria* to different *Corymbia* taxa: *dark grey* = highest, *white* = lowest, *light grey* = intermediate, *stippled* = no differences. These rankings were statistically significant except where otherwise shown

	CCV	CT × CCV	CT
leaf thickness			
specific leaf weight			
leaf glabrousness			
moisture content		*all equal*	
chemical profile			
field incidence			
adult feeding pref (lab)			
larval mortality rate (lab)	*a*	*ab*	*b*
overall larval mortality (lab)			
larval development time (lab)		*all equal*	
pupal weight (lab)			

compounds produced by juvenile foliage of shining gum, since larvae develop just as well on this foliage type as on adult foliage on which adults feed and oviposit. There often is not a tight linkage between paropsine oviposition preference and larval performance (Carne 1966; de Little and Madden 1975; Baker et al. 2002; Nahrung and Allen 2003), and since larval habitat is determined principally by the placement of eggs by females, oviposition preference is probably a more pertinent indicator of the plant attributes used for host selection.

Assuming that *P. atomaria* adult feeding preference reflects oviposition preference, larvae would rarely encounter CT under field conditions. Larval development time was unaffected by rearing host in these trials, but subsequent pupal mass was significantly lower on the hybrid. Increased pupal weight confers increased adult fecundity in *P. atomaria* (Carne 1966), suggesting a reduction in herbivore fitness arising from larvae developing on the hybrid. This result cannot be attributed to foliar physical characteristics, since the hybrid displayed intermediate or CT-equivalent traits, but there were five chemical components detected only in CT × CCV foliage, including 1,8-cineole, α-cubebene, and β-patchoulene, which may have contributed physiologically to lower pupal weight. 1,8-Cineole and α-cubebene were detected in very low amounts in CCV foliage (Asante et al. 2001), and were probably present in trace amounts in parental foliage here, representing an additive (*sensu* Fritz et al. 1999) effect in the hybrid.

Overall larval mortality was highest on *P. atomaria*'s sympatric host, CCV, and we attribute this to the high SLW and lamina thickness in this species: leaf toughness impedes feeding establishment of neonate paropsine larvae (Ohmart et al. 1987; Larsson and Ohmart 1988; Nahrung et al. 2001); yet, unexpectedly, larvae were able to feed on the densely hairy CT foliage. Leaf trichomes are a deterrent to herbivory in many plant species (Kitamura et al. 2007; Bjorkman et al. 2008). *Paropsis atomaria* larval growth rate also appears unaffected by tannins and other phenolic compounds in host foliage (Fox and Macauley 1977): larvae absorb terpenoids and probably have a metabolic detoxification process for dealing with them (e.g., 86% of ingested 1,8-cineole was absorbed or converted to other compounds) (Ohmart and Larsson 1989).

Although hybrid susceptibility to herbivores is predicted in eucalypts (Dungey and Potts 2003; Potts and Dungey 2004), the hybrid taxon displayed intermediate susceptibility (field incidence) to *P. atomaria* in our study, as it did with respect to possum damage (Scott et al. 2002). Nevertheless, our results suggest a possible chemical basis for host selection behavior and that selection for potential resistance may be possible for this species (see also Henery et al. 2008). Differential resistance to a number of significant insect pests, including eucalypt weevil (Dungey and Potts 2003), sawflies (Jordan et al. 2002), leaf beetles (Raymond 1995; Rapley et al. 2004a), and autumn gum moth (Jones et al. 2002; Rapley et al. 2004b) has been found in other eucalypts.

Although we have detected useful chemical characteristics to distinguish among the taxa in this study, whether any of these are relevant to the host finding/acceptance behavior of *P. atomaria* is unclear. The next step is an examination of electroantennagraphic (EAG) responses of the beetle to the plant extracts, as a method of determining the cues used by beetles. Understanding the preferences of this insect pest will assist in the choice of parental taxa and hybrids used for forestry, as these hardwoods become an ever more important component of the industry.

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Appendix 1

Retention times, kovats retention index and tentative identities of components detected in hexane extracts of *Corymbia* leaves, and the percentage of replicates of each taxon in which the component was identified. Unidentified components are designated "?"

Ret. Time (min)	Kovats Index	Name	CCV	CT × CCV	CT
3.566	901	(*E*)-2-hexenal	0	0	100
3.614	905	*m*-xylene	100	100	0
4.339	956	α-pinene	100	100	100
5.064	999	β-pinene	80	100	100
5.398	1023	1,2,3-trimethyl benzene	80	100	100
5.87	1054	?	60	0	0
5.93	1058	limonene	80	100	0
6.146	1071	3-carene	20	100	100
6.297	1080	1,8-cineole	0	60	0
6.349	1083	?	60	0	0
6.788	1111	?	100	20	40
7.703	1176	?	20	40	0
7.974	1193	?	40	0	0
8.483	1232	?	60	0	0
9.109	1278	?	20	0	0
9.589	1314	hydrocarbon	20	100	100
9.937	1342	methyl naphthalene	80	100	100
11.109	1437	cycloisolongifolene	40	80	0
11.232	1447	4,11,11-trimethyl-8-methylenebicyclo[7,2,0]undec-4-ene	100	100	100
11.443	1465	alloaromadendrene	40	60	0
11.604	1478	α-cubebene	0	60	0
12.12	1523	β-patchoulene	0	20	0
12.16	1527	sesquiterpene	100	40	80
12.537	1561	1,2,3,4,6,8a-hexahydro-1-isopropyl-4,7-dimethylnaphthalene	80	80	0
12.846	1588	elemol	100	0	0
13.46	1646	sesquiterpene	100	100	100
13.622	1662	1,2,6-hexanetriol	60	80	80
13.737	1673	?	80	40	0
13.83	1681	?	0	20	0
13.94	1691	?	80	40	0
13.975	1695	?	80	40	0.
14.143	1711	sesquiterpene	100	0	0
14.207	1718	?	0	80	60
16.042	1906	oxygenated hydrocarbon	80	100	100
16.942	2003	? (N-containing)	60	100	100
17.377	2055	octadecanol	0	20	100
18.517	2192	? (N-containing)	0	40	80
19.171	2275	?	80	20	20
20.664	2472	?	0	40	0
20.713	2478	hydrocarbon	60	100	100
20.851	2496	hydrocarbon	60	100	100
20.885	2501	hydrocarbon	0	20	80
21.92	2633	?	0	100	100
22.253	2674	hydrocarbon	20	100	80
22.385	2690	hydrocarbon	0	100	100
23.252	2794	hydrocarbon	60	100	100

23.315	2802	hydrocarbon	40	80	100
23.524	2826	hydrocarbon	80	100	100
23.74	2851	?	0	20	80
23.834	2862	?	40	0	0
24.519	2939	hydrocarbon	100	100	100
24.833	2974	?	20	0	0
24.868	2978	?	20	0	0
25.006	2993	?	20	20	0
26.149	3115	eicosane	100	100	100
28.568	3357	?	0	0	20
28.896	3388	?	20	0	0
28.916	3390	?	20	0	0

Appendix 2

Mean ± s.e. percentage area under the peak for compounds (identified by retention time) used to distinguish between pairs of taxa (A) CCV vs. CT; (B) CCV vs. CT × CCV; (C) CT vs. CT × CCV

a

Retention time	Mean % area-CCV	Mean % area-CT	% contribution to group dissimilarity
12.846	32.3±7.89	0	6.68
3.566	0	5.51±1.07	4.34
23.252	1.30±1.10	16.6±1.62	4.17
14.143	4.34±1.07	0	4.02
5.93	12.7±5.54	0	3.81
24.519	3.37±2.07	33.8±2.47	3.59
26.149	1.50±0.61	25.0±2.02	3.47
3.614	1.93±1.14	0	2.98
22.385	0	1.03±0.18	2.87
23.524	0.43±0.19	5.14±0.75	2.49
4.339	22.5±8.18	3.33±1.24	2.42
13.46	5.60±1.20	0.21±0.05	2.39
17.377	0	0.44±0.10	2.28
21.920	0	0.38±0.03	2.25
19.171	1.14±0.72	0.01±0.01	2.18
6.146	1.14±1.14	0.34±0.11	2.15

b

Retention time	Mean % area-CCV	Mean % area-CT × CCV	% contribution to group dissimilarity
12.846	32.3±7.89	0	7.70
14.143	4.34±1.07	0	4.64
23.252	1.30±1.10	10.5±0.65	4.07
5.93	12.7±5.54	0.56±0.03	3.29
6.146	1.14±1.14	1.49±0.38	3.17
20.851	0.15±0.06	3.02±0.35	3.01
24.519	3.37±2.07	18.1±1.70	2.97
20.713	0.17±0.07	2.75±0.28	2.86
22.385	0	0.53±0.14	2.76
21.923	0	0.45±0.16	2.71
19.171	1.14±0.72	0.04±0.04	2.45
22.253	0.06±0.06	0.57±0.08	2.43

26.149	1.50±0.61	9.34±0.53	2.40
23.524	0.43±0.19	3.32±0.31	2.36
6.788	0.53±0.13	0.03±0.03	2.37
8.483	1.24±0.53	0	2.28
c			
Retention time	Mean % area-CT	Mean % area-CT × CCV	% contribution to group dissimilarity
3.566	5.51±1.07	0	7.72
3.614	0	4.08±0.59	7.18
4.339	3.33±1.24	30.5±1.24	5.46
5.93	0	0.56±0.03	4.42
13.46	0.21±0.05	4.94±0.75	4.14
17.377	0.44±0.10	0.11±0.11	3.37
23.74	0.39±0.17	0.14±0.14	2.89
12.537	0	0.21±0.07	2.84
20.851	0.38±0.09	3.02±0.35	2.76
20.885	0.24±0.07	0.13±0.13	2.70
20.713	0.39±0.11	2.75±0.28	2.63
11.109	0	0.13±0.05	2.51
26.149	25.04±2.02	9.34±0.53	2.48

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To date, management of *L. botrana* in vineyards is based mainly on insecticidal control of preimaginal stages during the second generation of the year (Ioriatti et al. 2009), even though preventive strategies, such as pheromone mating disruption, are increasingly and effectively used in Area Wide Projects (Ioriatti et al. 2008). In addition to pheromones, substantial research has been directed at finding efficient attractants for grapevine moth females because such attractants could improve the timing of management decisions. Fermented fruit juices currently are considered useful for monitoring numbers of both sexes of *L. botrana*, but their use is time-consuming because the captured insects must be handled, sorted, and identified (Thiéry et al. 2006).

L. botrana females also were attracted to essential oil of rosemary (*Rosmarinus officinalis* L.) in Greece (Katerinopoulos et al. 2005), and to tansy flowers (*Tanacetum vulgare* L.) in a Slovakian vineyard (Gabel 1992); the steam-distillate extract from tansy flowers and a synthetic blend based on that extract consistently attracted females in field cages (Gabel et al. 1992).

Susceptibility to oviposition by *L. botrana* females, as well as larval fitness, differs among varieties of *Vitis vinifera* L. (Fermaud 1998; Snjezana 2004; Moreau et al. 2006; Sharon et al. 2009). Finding a good site for oviposition is a challenging task for female grapevine moths, and their decision has a critical consequence for the life history of the offspring. For this reason, *L. botrana* females probably rely not on a single cue but rather on a combination of visual, mechanical, and chemical properties of the host plant. The contribution of olfactory cues in determining the host plant search image is not well understood (Städler 2002), and it is still unknown whether secondary metabolites emitted by grape bunches carry specific information about their suitability as oviposition substrates and food sources for the offspring. According to recent reports, *L. botrana* females use contact cues to evaluate the suitability of substrates for oviposition (Maher et al. 2006); moreover, they are attracted from long distances to odors either released from grape clusters (Tasin et al. 2005, 2006a, 2006b, 2007; Masante-Roca et al. 2007) or grape leaves and flower buds (Masante-Roca et al. 2007).

Our hypothesis is that mated grapevine moth females, after finding and selecting the host plant, also use olfaction at a closer range as a first step in the complex chain of behavioral responses to cues for discriminating suitable oviposition sites. Preliminary laboratory bioassays have shown that odor emitted from grape bunches of different varieties is able to drive the oviposition at a short range of both *L. botrana* females (Tasin et al. 2008, 2009) and the related species *Eupoecilia ambiguella* (Hübner) (Anfora et al. 2008). To verify this hypothesis, we studied how *L. botrana* oviposition was affected by the emission of grape clusters of the varieties Trebbiano and Sangiovese. These varieties, which are commonly grown in Italy and especially in the Tuscany region, were selected because they differ in susceptibility to *L. botrana* infestation under natural conditions. Mainly during the second *L. botrana* flight of the year, Sangiovese was shown to be much more susceptible than Trebbiano (Bagnoli 1990). Hence, a further aim was to establish whether olfaction plays a role in such a varietal different susceptibility.

To date, most behavioral studies on grapevine moth females have been carried out in the laboratory with fresh and synthetic material. No data are available on the attractiveness of synthetic host plant volatiles to females under field conditions, where the odor competes strongly with the canopy background (Cha et al. 2008b).

In the current study, a lure blended with the six major electrophysiologically active volatile compounds released by either Sangiovese or Trebbiano was first tested in the laboratory for its attractiveness to *L. botrana* females and its effect on oviposition. Then, traps baited with this mimic were used in a field cage placed in a vineyard, and the effect on female behavior was evaluated in terms of trap captures and oviposition preference.

Methods and Materials

Insects Moths were selected from a colony of *L. botrana* reared on an artificial diet at the IASMA-FEM Research Centre. During experiments, wild larvae were periodically collected from Tuscan vineyards and added to the colony to reduce inbreeding. Insects were reared under a 18:6 h L:D photoperiod and at 22°C. Oviposition assays and trapping tests were done with 48 to 96-h-old mated females, individually placed overnight in a little cylindrical cage (3 cm ID×5 cm) to ascertain the beginning of oviposition.

Plant Material and Collection of Volatiles Grape clusters of *V. vinifera* Sangiovese and Trebbiano varieties were collected from an experimental vineyard at Pisa University (Colignola, Italy, 43° 44′ N, 2° 0′ O) from May to September 2006. Grapevines were sprayed with fungicides to control powdery and downy mildew. No insecticides were used during the trial. The grape clusters were collected at five different phenological stages (stage 17, fully developed inflorescences; stage 23, full flowering; stage 33, beginning of berry touch; stage 35, beginning of ripening; and stage 38, berries ripe for harvest) (Eichhorn and Lorenz 1977). Per each extraction, 25 inflorescences were collected from different plants for stages 17 and 23 (total ca. 150 g), and 3 grape clusters were collected for stages 33 (total ca. 600 g), 35, and 38 (1,000 g, respectively). The cut stem of each inflorescence or grape cluster was sealed with liquid paraffin, and the material was stored in a portable refrigerator at 4°C

for transfer to the nearby laboratory. There, inflorescences or grape clusters were placed immediately in a 25×38 cm polyacetate bag (Toppits, Melitta, Sweden) for collection of volatiles (Faccoli et al. 2008). Air from the headspace of each bag was drawn at 150 ml/min through a sorbent cartridge (75 mg Super Q; Sigma-Aldrich, Milan, Italy) connected to a vacuum pump. Charcoal-filtered air was pulled back simultaneously into the bag by the same pump to maintain a constant pressure. Volatiles were collected for 6 h. Volatiles were eluted from the sorbent cartridge by solvent desorption at room temperature using 500 µl of hexane (>99% purity, Sigma-Aldrich). Five to six collections from different groups of inflorescences or grape clusters were done for each variety in each phenological stage. Some of the extracts were prepared for chemical quantification, and 0.5 µg of heptyl acetate (purity≥99%) were added as an internal standard (Bengtsson et al. 2001). Collections were reduced to 50 µl by using a slow stream of nitrogen, and were stored in 2-ml vials at −18°C until use.

For stage 33, volatiles of the two varieties were also collected directly in the field using intact grape clusters attached to the plants. This was done to determine whether there were qualitative and/or quantitative differences in volatiles collected in the laboratory vs. the field.

Chemicals Limonene, (±)-linalool, (*E*)-caryophyllene, and methyl salicylate were purchased from Sigma-Aldrich and (*E,E*)-α-farnesene from Firmenich (Geneva, Switzerland). 4,8-Dimethyl-1,(*E*)-3,7-nonatriene [(*E*)-DMNT] was synthesized by Ingve Stenstrøm (Aas, Norway).

Chemical and Electrophysiological Analyses Chemical and electrophysiological analyses were performed using methods described by Anfora et al. (2005) and Faccoli et al. (2008). Collections of plant volatiles and blends of synthetic compounds were analyzed by a Hewlett Packard (Palo Alto, CA, USA) 5890 gas chromatograph with a flame ionization detector and an Innowax column (30 m×0.2 mm × 0.32 µm) programmed from 60°C (3 min hold) at 8°C/min to 220°C (10 min) coupled to a Syntech electroantennodetector (Hilversum, The Netherlands) (GC-EAD). The antennae were cut from the moth head and suspended between two glass electrodes filled with Kaissling solution (Kaissling 1987). The mounted antennae were placed into the air stream that carried the volatiles compounds eluting from the GC column. Each sample was analyzed three times.

Volatiles collected in headspace entrapments were identified by a Perkin-Elmer AutoSystem XL Gas Chromatograph (60 m×0.32×0.5 µm DB-Wax fused silica column, J&W Scientific Inc., Folsom, CA, USA; for temperature program, see GC program above) equipped with a mass spectrometer (Perkin-Elmer TurbomassGold) with an ionization potential of 70 eV. Helium at 1.2 ml/min was used as carrier gas (GC-MS). Splitless injection (2 µl) was used. The injector was programmed at 250°C, and the transfer line was set at 220°C. Identity of GC-EAD active compounds was verified by injections with synthetic standards. The mean antennal responses were calculated by means of further GC-EAD analysis, injecting blended synthetic compounds at the constant amount of 10 ng of each one.

Laboratory Oviposition Bioassay The oviposition preference of *L. botrana* females for volatiles emitted by grape clusters of Sangiovese vs. Trebbiano was assessed with a dual-choice oviposition assay (Anfora et al. 2008; Tasin et al. 2009). Oviposition assays were conducted in ventilated cylindrical cages (25 cm ID×45 cm; metallic net, 2-mm mesh). Each cage contained two plastic and transparent conical glasses (bottom 61 mm, top 88 mm, height 130 mm), which were located 7 cm below the cage top and 10 cm from the cage sides (the glasses were separated by 25 cm). Each glass had 30 holes (1.5±0.2 mm ID) to allow volatiles to escape; one glass contained one grape cluster of Sangiovese and the other contained one cluster of Trebbiano; in both cases, the clusters were at phenological stage 33 (beginning of berry touch). At dusk, one mated female was released into the center of each cage. After 72 h at 25°C and 16:8 h L:D photoperiod, the moth was removed, and the eggs laid on glasses were counted and expressed either as the percentage of the total number of eggs laid on each glass or as the number of eggs laid per female in each cage. In this assay, the female moth's choice of oviposition site was based exclusively on visual and olfactory cues and not on contact chemoreception. This assay was replicated 18 times.

The dual-choice oviposition assay also was conducted with the synthetic lure (see Results). The synthetic lure was loaded in red rubber septa (Wheaton, 20 mm straight plug stopper, Millville, NJ, USA) at 15, 150, or 1,500 µg per septum. Septa were placed in a hood for 2 h to permit solvent evaporation (Heath et al. 1986), and subsequently were inserted into the conical glasses. One glass in the cage contained a septum with lure and the other contained a septum initially treated with solvent without lure. Oviposition was quantified as described previously, and the experiment was repeated 15 to 21 times.

To detect the possibility of a positional bias in the dual-choice oviposition assay, a preliminary experiment was performed. Oviposition was quantified after moths were released into cages that contained two empty glasses; the glasses were located on opposite sides (designated East and West) of the cage. This experiment was repeated 19 times.

Field-cage Experiment A release-capture experiment in a field cage was conducted in June and July 2008 in a thin-

meshed net tunnel (length 30 m, width 4 m, median height 2.5 m, 2-mm mesh) that covered two grapevine rows (guyot training system) of a Chardonnay vineyard at the IASMA-FEM Research Centre (San Michele all'Adige, Italy, 46° 12′ N, 11° 8′ E). The Chardonnay vineyard was chosen with the aim of avoiding a full overlapping between the volatiles from Trebbiano and Sangiovese and the background odor. The canopy height was 2.5 m, with 0.8 m between vines and 3 m between rows. A piece of mesh was used to divide the tunnel into two 15-m-long cages. All grape clusters within the cages were removed to reduce competition with natural odors. In each cage, three replicates of three differently baited traps (nine traps total per cage) were installed 1 m above the ground along the two rows: four traps were in one row and five were in the other row, with 3 m separating traps in the same row. In preliminary field trials, different trap models were evaluated for their suitability to catch females (data not shown). The traps employed consisted of square sticky base (30×30 cm) with a clear plastic cylindrical screw-top jar (8 cm ID× 11 cm height) in the middle. Thirty holes (1.5±0.2 mm ID) in the side wall of each jar allowed volatiles to escape. The screw top of each jar was fixed to the sticky base. The jar was unscrewed from its top to introduce the bait.

Three baits or treatments were compared: grape mimic, a cut grape bunch (phenological stage 33, Chardonnay from the same rows used in the experiment), and a blank. For the grape mimic, the solution (15 mg diluted in an equal volume of light mineral oil, Sigma-Aldrich) was impregnated in a rubber septum (Wheaton, 20 mm straight plug stopper). This formulation was chosen, as from the one set up in the laboratory bioassay, on the basis of preliminary tests with the aim of providing an adequate emission in field conditions and to prevent earlier evaporation of volatiles. Mated females (N=140) were released every 2nd week between the rows of each cage, for a total of three releases per cage. The releases alternated between cages so that only one cage received females in any week. At each release, the position of the traps was randomized and the grape mimic baits were replaced. The cut grape bunches were replaced every day. The number of females captured on the sticky base of the traps was determined daily for the 1st week after release, and the number of captures over the 7-day period was considered. Four weeks after the first release in each cage, *L. botrana* larvae were counted on grapevine shoots and leaves within 50 cm of each trap. Counting lasted 5 min/replicate, and presence of larvae developing in shoots and leaves was ascertained.

Statistical Analyses The software used for the statistical analyses was KyPlot 5.0 (Kyenslab Inc., Tokyo, Japan). The *Wilcoxon test* for paired samples was used to determine significant differences in the numbers of eggs laid between the two glasses in the dual-choice laboratory bioassay. Nonparametric *one-way ANOVA* (*Kruskal-Wallis test*) followed by the *Bonferroni-Dunn post-hoc multiple comparison test* was used to assess differences either in the number of eggs laid per female for the laboratory assays or in the attractiveness of the baits for the field-cage experiment. Parametric *one-way ANOVA* followed by the *Tukey post-hoc multiple comparison test* was used to assess the effect of baits on the level of pest infestation; homogeneity of variance had been determined previously with *Levene's test*.

Results

Chemical and Electrophysiological Analyses Antennae of grapevine moth mated females responded to 20 compounds from headspace collections of the two grape varieties (Table 1). The two odor profiles perceived by mated females had a high degree of similarity. Fifteen GC-EAD-active volatiles were released by both varieties; pentadecane, nonanal, decanal, and (*Z,E*)-(α)-farnesene were detected only from Sangiovese, while germacrene-(*D*) was detected only from Trebbiano.

For both varieties, several terpenes, released in all phenological stages and frequently in large amounts, elicited the highest antennal responses. The most abundant terpenes were (*E*)-DMNT at stage 17 and limonene from stage 33 to stage 38. (*E*)-DMNT, limonene, (*E,E*)-α-farnesene, (±)-linalool, (*E*)-caryophyllene, and methyl salicylate always were collected during the three grape phenological stages when *L. botrana* oviposition occurs in nature (stages 17, 33, and 35). The average amounts of emission of these terpenes are shown in Table 1. Synthetic (*E*)-DMNT elicited the strongest antennal responses in *L. botrana* mated females.

For stage 33, field-collected and laboratory-collected volatiles did not differ significantly qualitatively or quantitatively (data not shown). The chemical and electrophysiological data were used to formulate the synthetic lure.

Formulation of Synthetic Lure Since in the oviposition bioassay the volatiles emitted by the grape clusters of Trebbiano and Sangiovese did not cause a different attractiveness for *L. botrana* gravid females (see following section), a synthetic lure that approximated the emission of both varieties was prepared. The lure contained limonene, (*E*)-DMNT, (±)-linalool, (*E*)-caryophyllene, (*E,E*)-α-farnesene, and methyl salicylate. Such compounds were GC-EAD active, and always were collected from both varieties during the phenological stages 17, 33, and 35, corresponding to the three *L. botrana* flights of the year. The relative proportions of these volatiles change during the season according to the

Table 1 Relative quantities of volatile compounds, antennally active in GC-EAD experiments on *Lobesia botrana* females, collected in the headspace of bagged grapevine clusters belonging to varieties Trebbiano (T) and Sangiovese (S) at five phenological stages[a], and antennal responses to synthetic compounds[b]

Phenological stage[c]	17		23		33		35		38		
Compounds[d]	T	S	T	S	T	S	T	S	T	S	EAD (mV)
Hydrocarbons											
pentadecane		1									0.02±0.01
1-heptadecene					2	1					0.01±0.01
Alcohols											
1-octen-3-ol		<1		<1	1	<1		1		2	0.39±0.21
Aldehydes											
nonanal		14									0.04±0.02
decanal		9									0.03±0.02
Ketones											
2-tridecanone			31	17							0.01±0.01
2-pentadecanone			21	6							0.02±0.01
Aromatics											
methyl salicylate	1	2	<1	<1	6	3	4	1		<1	0.06±0.02
(*Z*)-3-hexenyl benzoate			<1	<1							0.04±0.02
Irregular terpenoids											
(β)-ionone			1	3							0.01±0.01
Monoterpenes											
limonene	70	72	2	3	100	100	100	100	100	100	0.07±0.03
(*E*)-DMNT	100	100	3	2	6	10	1	6			0.59±0.22
(±)-linalool	3	12	1	<1	4	4	2	1			0.03±0.01
Sesquiterpenes											
(*E*)-caryophyllene	7	39	44	41	5	15	4	4			0.07±0.03
(*E*)-(β)-farnesene		14	1	4	9	2					0.03±0.01
α-humulene			17	17	3	8		1			0.03±0.01
germacrene-(*D*)							34				0.04±0.02
(*Z,E*)-(α)-farnesene		2									0.07±0.03
(*E,E*)-(α)-farnesene	5	19	100	100	9	4	14	1			0.18±0.05
caryophyllene oxide	<1		3	2							0.07±0.02

[a] Quantities are relative to the most abundant compound (set at a value of 100) in each phenological stage

[b] Mean ± SD antennal responses (mV) using 10 ng of standard compounds (N=3)

[c] *Vitis vinifera* phenological stages according to Eichhorn and Lorenz (1977): 17 (fully developed inflorescences), 23 (full flowering), 33 (beginning of berry touch), 35 (beginning of ripening), and 38 (berries ripe for harvest)

[d] The average amount of the most abundant compound collected from 100 g of grape clusters in each phenological stage were: Trebbiano) 376.5± 167.4 ng/h of (*E*)-DMNT (fully developed inflorescences); 9,866.8±2,987.2 ng/h of (*E,E*)-(α)-farnesene (full flowering); 556±234 ng/h of limonene (beginning of berry touch); 80.2±19.9 ng/h of limonene (beginning of ripening); and 110.6±38.0 ng/h of limonene (berries ripe for harvest); Sangiovese) 89.6±56.8 ng/h of (*E*)-DMNT (fully developed inflorescences); 5,481.1±1,089.6 ng/h of (*E,E*)-(α)-farnesene (full flowering); 569.7±312.5 ng/h of limonene (beginning of berry touch); 54.6±27.1 ng/h of limonene (beginning of ripening); and 124.6±43.1 ng/h of limonene (berries ripe for harvest)

phenological stage of the grape. Because stage 33 corresponds to the second flight of *L. botrana*, and because the bunches at this phenological stage were used for the oviposition bioassay, the lure was formulated based on the proportions released from grapes at stage 33. Grape volatiles of stage 33 were found in the following ratios: 10:0.6:0.4:0.5:0.9:0.6 [limonene:(*E*)-DMNT:(±)-linalool:(*E*)-caryophyllene:(*E,E*)-α-farnesene:methyl salicylate] from Trebbiano and 10:1:0.4:1.5:0.4:0.3 from Sangiovese (Table 1). Because the components and their ratios were similar for the two varieties at stage 33, the synthetic lure was mixed with the six components in the approximate and simplified proportion

10:1:1:1:1:1 [limonene:(*E*)-DMNT:(±)-linalool:(*E*)-caryophyllene:(*E,E*)-α-farnesene:methyl salicylate]. Thereby, the aim was to mimic both varieties by using a lure easy to prepare. For laboratory and field bioassays, an hexane solution was prepared at the concentration of 100 μg/μl for limonene and 10 μg/μl for the other compounds, and it was used as a mother solution.

Laboratory Oviposition Bioassay In the dual-choice bioassay, oviposition (number of eggs laid per glass) was similar (T=60.0; N=18; P=0.44) for glasses containing cut grape clusters of Sangiovese and Trebbiano (Fig. 1). The effect of synthetic lure (grape mimic) on oviposition depended on lure concentration (Fig. 1). Grape mimic at 150 μg/septum induced oviposition (T=54.0; N=21; P<0.05); grape mimic at 1,500 μg/septum repelled oviposition (T=13.0; N=15; P<0.05); and grape mimic at 15 μg/septum did not affect oviposition (T=66.5; N=16; P=0.94). No bias related to the position of the glasses in the cage was detected (T=83.5; N=19; P=0.93). *L. botrana* females laid significantly more eggs (T=39.9; N=89; P<0.001) in the cages where either the grape bunches were present or the synthetic lure at the dosage of 150 μg/septum (Fig. 1).

Field-cage Experiment The recapture rate in the field-cage experiment was approximately 10% (83 individuals caught vs. 840 released). Traps baited with grape mimic caught significantly more mated *L. botrana* females than traps baited with one grape cluster or unbaited traps, and traps baited with grape clusters captured more females than unbaited traps (KW=7.2; df=2; P<0.05) (Table 2).

The numbers of grapevine moth larvae on shoots and leaves near the traps differed significantly with bait (F=59.0; df=15; P<0.001). The level of infestation was significantly higher near traps baited with synthetic lure than near unbaited traps or traps baited with grape clusters (Table 2).

Discussion

Two main goals motivated our research on olfactory communication between plant and moth: to understand the role of olfactory cues in the selection of oviposition sites by *L. botrana*, and to develop a synthetic lure based on host-plant volatiles for monitoring female flight activity in the field. Our first hypothesis was that olfactory cues could affect greatly the selection of oviposition sites among different host-plant genotypes. This was not confirmed by our results. The two varieties chosen for their different susceptibility to *L. botrana* did not show significant differences in odor profiles (volatiles released), and the volatiles from these two varieties elicited similar antennal responses. Moreover, the two odor profiles did not differ in their ability to attract ovipositing females in the dual-choice bioassay.

L. botrana is a generalist herbivore with a long list of potential host plants (Bovey 1966). Schoonhoven et al. (1998) argued that host-plant recognition for polyphagous insects should result largely from the lack of repellents rather than from the presence of specific attractants. On the

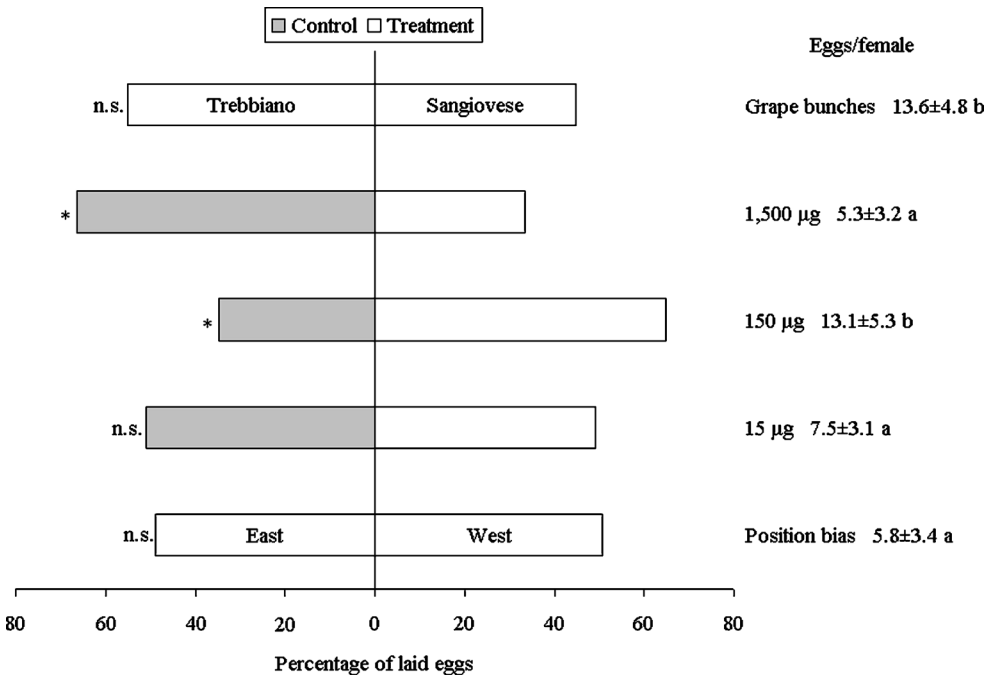

Fig. 1 Oviposition preference of gravid female *Lobesia botrana* toward Trebbiano and Sangiovese grape bunches at phenological stage 33 and a blend mimicking their released volatiles. The grape mimic was formulated to contain 15, 150, and 1,500 μg of compounds. *Asterisk* indicates significant deviation from equal preference in each experiment (P<0.05, n.s. = non significant, *Wilcoxon test* for paired samples). The mean number of eggs laid per female in each experiment is indicated in the *right* column. Values within the column followed by different letters are significantly different (P<0.001, *Kruskal-Wallis test* followed by *Bonferroni-Dunn post-hoc multiple comparison test*)

Table 2 Number of *Lobesia botrana* females captured in horizontal traps and number of *L. botrana* larvae dwelling inside grapevine leaves near the traps (within 50 cm) in the field-cage experiment[a]

	Number of females captured in traps[b]	Number of larvae near traps[c]
Grape mimic	6.3±2.6 c	9.8±0.8 c
Grape bunch	4.5±2.1 b	4.7±1.4 b
Blank	2.8±1.0 a	2.5±1.4 a
	KW=7.2; df=2; P<0.05	F=59.0; df=15; P<0.001

[a] Mean number ± SD of females captured in the traps (N=6) and larvae dwelling in grapevine tissues are shown; traps were placed in a thin-meshed net cage (length 30 m, width 4 m, median height 2.5 m) and baited with 15 mg of grape mimic or a grape bunch (phenological stage 33)

[b] Values within the column followed by different letters are significantly different (*Kruskal-Wallis test* followed by *Bonferroni-Dunn post-hoc multiple comparison test*)

[c] Values within the column followed by different letters are significantly different (*one-way ANOVA* followed by *Tukey post-hoc multiple comparison test*)

other hand, the absolute and relative amounts of ubiquitous plant volatiles have been suggested as the critical factors that mediate the species specificity of host-plant profiles (Visser 1986; Bruce et al. 2005) and, as a consequence, the differential attractiveness of varieties of a given species to certain herbivores. In our case, the ratio of the active volatiles, such as the most abundant limonene, was similar in both varieties at phenological stage 33 and thus cannot explain differences in their susceptibility to grapevine moth. The lower susceptibility expressed under natural conditions by Trebbiano vs. Sangiovese might be due to factors such as the asynchrony between susceptible phenological stage and moth flight (Bagnoli 1990), the ecological setting of the varieties, and the influence of other sensory cues (Maher et al. 2006; Moreau et al. 2006; Pavan et al. 2009). The characterization of the different sensory cues released by grapevine genotypes that differ in tolerance to grapevine moth could allow the development of innovative methods for manipulating *L. botrana* behavior.

Our synthetic lure mimic of both Trebbiano and Sangiovese attracted *L. botrana* egg-laying females either in the laboratory bioassay or in the field-cage experiment. From a comparison of the headspace collections from Chardonnay grape variety (Tasin et al. 2005) and of the blend attractants from Casana in a wind tunnel bioassay (Tasin et al. 2006b, 2007) with the set up from Trebbiano and Sangiovese, strong differences were shown in the active compounds and their ratio. In particular, we found in the collected volatiles a higher amount of limonene, which never was included in previous blends attractive to *L. botrana*. This confirms the remarkable differences that are commonly observed between cultivars and the plasticity of the moth in host recognition. It is noteworthy that, in the absence of grape bunches in the field cages, *L. botrana* females deposited their eggs on grapevine shoots and leaves, and that the larvae seemed capable of developing inside such organs. This is in agreement with the reported attractiveness of various grapevine plant parts to *L. botrana* in wind tunnel experiments (Tasin et al. 2005; Masante-Roca et al. 2007). Furthermore, Cha et al. (2008a) demonstrated that grape shoot tips and mature leaves were more attractive than unripe and ripe berries or flowers to females of *Paralobesia viteana* Clemens. Based on their analysis of the shoots of *Vitis riparia* Michx, the same authors formulated a synthetic blend that, when used as bait in traps, caught females of *P. viteana* in a vineyard (Cha et al. 2008b).

Besides attractiveness, plant volatiles are able to enhance oviposition of *L. botrana*, as in codling moth (Witzgall et al. 2005). In the oviposition bioassay, gravid females laid significantly more eggs in the presence of the odor either from the grape bunches or from the synthetic lure at the attractive dosage. Moreover, the large number of *L. botrana* larvae detected near traps baited with synthetic lure in the field bioassay corroborates our hypothesis that *L. botrana* uses olfaction to select oviposition sites, as well as the volatiles from grape bunches that likely stimulate its egg-laying behavior.

Based on our results, we conclude that *L. botrana* uses a subset of common volatiles emitted by grape clusters both to find hosts and to select oviposition sites. In this way, the behavior of *L. botrana* is similar to that of other tortricids (Wearing and Hutchings 1973; Suckling et al. 1996; Grant et al. 2007). On the other hand, the plasticity in the responses of *L. botrana* to ubiquitous plant compounds at different ratios can also explain the keeping of several plant species and grape varieties as hosts that provide different fitness (Thiéry and Moreau 2005; Tasin et al. 2007).

The lesser attractiveness of cut Chardonnay grape cluster bait vs. synthetic grape mimic bait could be due to strong competition with the natural odor in the Chardonnay vineyard, despite the removal of grape clusters on the plants within the cages. However, because the attractiveness of the grape mimic recorded in the field cage was quite

weak, we assume that its attractiveness also can be affected by the vineyard background odor. In a given environment, each plant is capable of synthesizing ten to hundreds of primary and secondary metabolites, with diverse biological properties and functions that can create complex backgrounds able to disrupt insects from selecting acceptable host plants or synthetic blends (Knudsen et al. 2008). Consequently, an artificial lure for capturing females must be able to compete with background odors. Furthermore, the approximate ratio of compounds in the blend, based on our specific extraction conditions, along with the release rates of dispensers, are both likely to be considerably discrepant from natural emissions of grapes and affect negatively the attractiveness of the lure (Heath et al. 1986; Tasin et al. 2005; Cha et al. 2008b), as the results of the oviposition bioassay suggest. In fact, the interference of the grape mimic with the *L. botrana* egg-laying behavior was dose-dependent, either attracting oviposition or inducing an antioviposition activity at a higher dose. Hence, future studies have to include the optimization of the blend rate in the field.

We agree with Bernays and Chapman (1994) that the role of odors in host location is a stronger selective force for specialists than for generalists. Nevertheless, results of our experiments provide new evidence that plant volatiles play a relevant role in all three-linked events of the host plant selection process: host finding, landing on the proper plant organ, and egg laying stimulation. Such a situation might drive the species toward a long-term specialization in host recognition.

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1978; Hiwot 2000). Fermentation products, e.g., residue (dregs) from tella, an Ethiopian beer-like beverage, are highly attractive to the beetles (Ministry of Agriculture and Ethiopian Agricultural Research Organization 1999). Tella is a spontaneously fermented beer (no yeast is added) that is brewed using water, flour of sorghum or other cereals, malt of barley or wheat, with crushed leaves of shiny-leaf buckthorn, *Rhamnus prinoides* Eschsch. (Rhamnaceae), instead of hops. *Pachnoda interrupta* adults also feed on the herbaceous weed abutilon, *Abutilon figarianum*, and food crops such as pearl millet, *Pennisetum glaucum* and sorghum, *Sorghum bicolor* (Schmutterer 1969; Grunshaw 1992; Jago 1995; Sastawa and Lale 2000). During the early 1990s, the sorghum chafer emerged as a key pest on sorghum in Ethiopia (Hiwot 2000). Mean percent loss of sorghum yield due to *P. interrupta* infestation can be as high as 70% (Yitbarek and Hiwot 2000). Efficient control methods for this pest insect are lacking, but trapping by using fruit (mainly *Musa* spp.) as bait shows promise for decreasing the pest population (Ministry of Agriculture and Ethiopian Agricultural Research Organization 1999). The efficiency of trapping could be improved by using better traps and synthetic, standardized attractants (Wolde-Hawariat et al. 2007). Previous field experiments indicate that compounds commonly found in the odor profile of flowers and fruits have potential as attractants for *P. interrupta*, with high levels of attraction especially to methyl salicylate and eugenol (Wolde-Hawariat et al. 2007). Single compounds are efficient lures for many scarab species that feed on fruits and flowers (Donaldson et al. 1990), and studies have shown a high degree of overlap between active compounds identified from different food sources (Stensmyr et al. 2001). Continued efforts in field testing of novel synthetic attractants for the Japanese beetle, *Popillia japonica*, have led to the development of lures far outperforming initial versions (Potter and Held 2002).

In search of attractants for pest insects, various approaches have been employed. Trial-and-error field screening of compounds has led to the identification of powerful attractants, e.g., for *P. japonica* (Potter and Held 2002). A commonly employed method in search of field attractants is gas chromatography with electroantennographic detection (GC-EAD; Arn et al. 1975), which allows the identification of antennally active compounds in the volatile blends emitted by hosts. GC-EAD has led to the identification of several powerful kairomonal field attractants (see e.g., Linn et al. 2005). Few studies, however, have employed single sensillum recordings (SSR) to identify behaviorally active compounds (Stensmyr et al. 2001; Larsson et al. 2002, 2003; Ulland et al. 2008), even though SSR may be a highly sensitive tool in detecting physiologically active components in the volatile profiles of host plants (Wibe 2004), including compounds that do not elicit detectable GC-EAD responses (Blight et al. 1995; Wu et al. 1995; Barata et al. 2002). SSR usually has been employed as a means of describing and understanding the sense of olfaction, specifically the olfactory receptor populations of antennae and palps (see e.g., Larsson et al. 2001; Mustaparta 2002).

We employed GC-EAD on volatile collections from sorghum and abutilon to identify compounds responsible for attraction of *P. interrupta* to these plants in the field. We also screened olfactory receptor neurons (ORNs) on the sorghum chafer antenna with potential kairomones using SSR. The behavioral activity of several compounds active in GC-EAD and SSR was tested in field trials, in search of potent attractants. Such attractants could be used in future control efforts, either in mass trapping or in monitoring, or as part of integrated pest management.

Methods and Materials

Insects for Electrophysiological Experiments Male and female sorghum chafers were collected at Rasa (09°55′N, 40°05′E), located 255 km northeast of Addis Ababa, Ethiopia. Adult beetles were sexed based on the presence of a ventral, abdominal groove in males (Rigout 1989), and kept separately. After transport to Alnarp, Sweden, adults were kept in clear plastic boxes (30×12×22 cm, Cofa Plastics AB, Stockholm, Sweden) with a 1:1:1 mixture of planting soil (Yrkesplantjord, Weibull Trädgård AB, Hammenhög, Sweden), peat (Växa trädgårdstorv, Econova Garden AB, Åse, Sweden) and composted cow dung (Simontorps Bas, Weibull Trädgård AB). Boxes were kept at 25°C, 70% relative humidity, and a L16:D8 h cycle. The beetles were fed with apples and bananas *ad libitum*.

Headspace Plant Volatile Collection Volatiles were collected from the developmental stage of the plant most attractive to the beetles, during the time of day when the beetles feed intensively, i.e., 10 am to 4 pm. For abutilon, the top 20 cm of a single abutilon plant, including flowers, seed pods, and leaves, was enclosed for each collection. For sorghum, volatiles were collected from a single panicle at the soft dough stage (milky stage). Polyacetate bags (35×43 cm; Toppits Scandinavia AB, Sweden) sealed with steel wire around the stem of the plant were used for aerations. An activated charcoal filter was placed next to the stem to filter incoming air. Volatiles were collected on glass tube columns (3.5 mm i.d.× 50 mm) packed with 25 mg SuperQ®, mesh 80/100 (Alltech, Deerfield, IL, USA) with glass wool and Teflon stoppers at both ends (Birgersson and Bergström, 1989). The filter was placed in the polyacetate bag and connected by PVC tubing to a small battery operated pump (PAS-500 Personal Air Sampler, Supelco, Bellefonte, PA, USA). The flow of the pump was 200 ml/min, and collections were made in the field

for 2 h. Immediately after collection, the columns were rinsed with 200 µl of redistilled hexane into 1.1 ml tapered glass vials (1.1 STVG, Chromacol Ltd., Welwyn Garden City, UK). Vials with extracts were kept in an icebox for transportation to the laboratory, and thereafter kept at —18°C until analysis.

Gas Chromatograph-Coupled Electroantennographic Detection (GC-EAD) The response of *P. interrupta* antennae to volatiles was studied by GC-EAD using an Agilent Technologies gas chromatograph (GC), model 6890, equipped with a fused silica capillary column (30 m×0.2 mm) coated with Innowax (0.25 µm film thickness) (Agilent Technologies Inc., Santa Clara, CA, USA). For each run, 2 µl of sample were injected in splitless mode. Hydrogen was used as mobile phase at a linear velocity of 45 cm/sec. The oven temperature was programmed from 40°C (5 min hold) to 230°C at 5°C/min. Compounds eluting from the column were split 1:1 in a four-way splitter, with nitrogen as make up gas (20 ml/min), and delivered to the FID and to the antenna. Compounds were carried to the antenna through a glass tube by a charcoal-filtered and humidified air stream at 0.5 m/sec. Antennae were mounted according to Leal et al. (1992) and Wolde-Hawariat et al. (2007). The antenna was excised with fine forceps and placed in an antennal holder (Hillbur 2001; JoAC, Lund, Sweden), and the signal was amplified (JoAC) and analyzed with GC-EAD software (Syntech, Hilversum, The Netherlands). EAD responses to FID peaks were defined as repeatable deflections of the baseline. Each extract was tested on five different antennae per sex, for a total of ten antennae per extract.

Chemical Identification Samples of plant volatile collections were analyzed by combined gas chromatography and mass spectrometry (GC-MS) Hewlett Packard 6890 GC and 5973 MS (Agilent Technologies Inc.). Extracts were injected with an HP 7683 auto injector in splitless mode. The GC was fitted with the same column under the same conditions as for GC-EAD, but usinig helium (35 cm/sec) as carrier. Peaks were matched between GC-EAD and GC-MS by retention index. Identifications of compounds were confirmed by comparison of mass spectra in the NIST 1998 and Wiley 1998 commercial mass spectral databases, and with those of authentic GC standards, except for methyl anthranilate, which was not available at the time when GC-MS analysis was done.

Synthetic Compounds Synthetic standards for all experiments were purchased from Sigma-Aldrich (for purity and CAS number, see Table 1). A total of 82 compounds were used in single sensillum recordings (Table 1). The compounds include volatiles commonly found in flowers (Knudsen et al. 2006), volatiles from tropical fruit (Macku and Jennings 1987; Ibáñes et al. 1998; Boudhrioua et al. 2003; Carasek and Pawliszyn 2006; Clara et al. 2007; Pandit et al. 2009), and volatiles related to microbial degradation and fermentation (Chatonnet et al. 1992; Fischer et al. 2000; Xiao and Ping 2007). Approximately half of the compounds used previously have been found to elicit behavioral or electrophysiological activity in the sorghum chafer or related scarab beetles (Stensmyr et al. 2001; Larsson et al. 2003; Wolde-Hawariat et al. 2007).

Single Sensillum Recordings (SSR) Single synthetic compounds were diluted to 1 µg/µl in acetone or hexane, depending on polarity (Table 1). Highly volatile compounds were diluted to 1 µg/µl in paraffin oil. Blends of 2–10 compounds, with each component at the same concentration as in the single compound dilutions, were also prepared for screening purposes (see below; Table 1). Stimuli were prepared by applying 10 µl of 1 µg/µl solution to a 1.5× 1 cm piece of Whatman filter paper (No. 3, Whatman, Maidstone, United Kingdom) that was placed in a disposable Pasteur pipette (150 mm soda lime glass, VWR International, Stockholm, Sweden). For compounds diluted in hexane or acetone, solvent was allowed to evaporate before stimuli were used in experiments. After evaporation of solvent, 1 ml pipette tips were put on the wide end of the Pasteur pipettes, to reduce any further evaporation of the test compound(s). Between trials, stimulus pipettes were kept at —18°C, to avoid evaporation. For comparison, stimulus pipettes containing only solvent as well as empty pipettes were prepared. To ensure that stimulus pipettes were not exhausted, new ones were prepared once per week (after having been used a maximum of ten times), except for screening pipettes, where new ones were prepared each day.

Insects were restrained with Parafilm (PM-992, Pecheney plastic packaging, Menasha, WI, USA) and fixed on microscope slides (ca. 76×26 mm, Menzel-Gläser, Braunschweig, Germany) using dental wax (Surgident periphery wax, Heraeus Kulzer GmbH, Hanau, Germany), with the lamellae held open on a wax surface using 2-3 mm long pieces of thin tungsten wire. A silver grounding electrode was inserted into the abdomen. Sensilla were contacted with a tungsten electrode (diam 0.12 mm, Harvard Apparatus Ltd, Edenbridge, United Kingdom) electrolytically sharpened in a saturated KNO_2 solution (Hubel 1957), using a DC-3K Rechts PM-10 piezo micromanipulator (Märzhäuser Wetzler GmbH, Wetzler, Germany). The signal from the ORNs was registered and amplified 10 times with a probe (INR-02, Syntech), amplified 200 times with a Syntech UN-06 AC/DC amplifier, and transferred to a computer through an IDAC-4-USB (Syntech), where it was visualized and analyzed with the software Autospike v. 2.2 (Syntech).

A constant flow of 0.5 m/sec of charcoal-filtered and humidified air was delivered through a glass tube with its outlet approximately 15 mm from the antenna. Stimuli were presented to the insect by inserting the stimulus pipette

Table 1 Synthetic compounds used for single cell screening

#	Compound	S	CAS	%
*1	4-Ethylphenol	A	123-07-9	99
*1	4-Methylphenol	A	106-44-5	99
*2	(*E*)-2-Hexenal	H	6728-26-3	98
*2	(*E*)-2-Hexen-1-ol	H	928-95-0	96
*2	(*E*)-2-Hexenyl acetate	H	2497-18-9	98
*2	(*E*)-3-Hexen-1-ol	H	928-97-2	98
*2	(*Z*)-3-Hexen-1-ol	H	928-96-1	98
*2	(*Z*)-3-Hexenyl acetate	H	3681-71-8	98
3	Hexanal	H	66-25-1	98
*3	1-Hexanol	H	111-27-3	98
*3	Hexyl acetate	H	142-92-7	98
*3	Nonanal	H	124-19-6	95
3	1-Nonanol	H	143-08-8	99,5
*3	1-Octanol	H	111-87-5	99,5
*3	(±)-3-Octanol	H	589-98-0	99
*3	(±)-1-Octen-3-ol	H	3391-86-4	98
*4	Anethole	H	4180-23-8	99
*4	Benzaldehyde	H	100-52-7	99,5
*4	Benzylalcohol	H	100-51-6	99
*4	Eugenol	H	97-53-0	98
*4	Methyl benzoate	H	93-58-3	99
4	Methyl anthranilate	H	134-20-3	99
*4	2-Phenylethanol	H	60-12-8	98
*4	2-Phenylethyl propionate	H	122-70-3	98
*5	(±)-Acetoin	A	513-86-0	97
*5	racemic 2,3-Butanediol	A	513-85-9	99
5	Carvacrol	A	499-75-2	98
5	Cinnamic aldehyde	A	104-55-2	98
*5	Methyl cinnamate	A	103-26-4	99
*5	Methyl salicylate	A	119-36-8	99
*5	Phenylacetaldehyde	A	122-78-1	90
*5	Phenylacetonitrile	A	140-29-4	99
*5	Thymol	A	89-83-8	99,5
*6	Butyric acid	H	107-92-6	99
*6	N-Caproic acid	H	142-62-1	99,5
*6	Isovaleric acid	H	503-74-2	98
6	Valeric acid	H	109-52-4	99,8
*7	Isoamyl alcohol	H	123-51-3	98
*7	6-Methyl-5-hepten-2-one	H	78-70-6	99
7	Tetradecane	H	629-59-4	99,5
7	Tridecane	H	629-50-5	99,5
*8	(±)-*beta*-Caryophyllene	H	87-44-5	98,5
*8	(-)-*trans*-Citronellol	H	106-22-9	95
*8	Geraniol	H	106-24-1	98
*8	Geranyl acetate	H	105-87-3	98
*8	(±)−Linalool	H	78-70-6	97
*8	Linalool oxides	H	n/a	97
8	Methyl jasmonate	H	1211-29-6	95
8	Nerolidol	H	7212-44-4	98
9	(±)−*delta*-Decalactone	H	705-86-2	98
9	(±)−*gamma*-Decalactone	H	706-14-9	97
9	(±)−*gamma*-Hexalactone	H	695-06-7	98
*9	(±)−*gamma*-Nonanlactone	H	104-61-0	97
9	(±)−*gamma*-Octalactone	H	104-50-7	97
9	(±)−*gamma*-Undecalactone	H	104-67-6	99
10	(±)−Ethyl 3-hydroxybutyrate	H	5405-41-4	97
*10	(*Z*)-3-Hexenyl butyrate	H	16491-36-4	98
*10	(*Z*)-3-Hexenyl isobutyrate	H	41519-23-7	98
*10	(*Z*)-3-Hexenyl tiglate	H	67883-79-8	97
*11	Butyl butyrate	H	109-21-7	98
*11	Ethyl butyrate	H	105-54-4	99
*11	Ethyl hexanoate	H	123-66-0	99
11	Ethyl propionate	H	105-37-3	99
*11	Hexyl butyrate	H	2639-63-6	98
11	Methyl butyrate	H	623-42-7	99
*11	Methyl hexanoate	H	106-70-7	99
*11	Methyl octanoate	H	111-11-5	99
11	Methyl propionate	H	554-12-1	99
*11	Propyl butyrate	H	105-66-8	99
12	Butyl isobutyrate	H	97-87-0	97
12	Hexyl hexanoate	H	6378-65-0	97
*12	Isoamyl acetate	H	123-92-2	98
*12	Isoamyl butyrate	H	106-27-4	98
12	Isobutyl acetate	H	110-19-0	99,8
*12	Isobutyl isobutyrate	H	97-85-8	99
12	Isopentyl isobutyrate	H	2050-01-3	98
*12	Isopropyl acetate	H	108-21-4	99,8
13	Acetic acid	P	64-19-7	99
13	Acetone	P	67-64-1	99,9
13	Ethanol	P	64-17-5	99
*13	Ethyl acetate	P	141-78-6	99,5
13	Propionic acid	P	79-09-4	99,5

*, compound active in single sensillum recordings

#, screening blend

S, solvent used (A, acetone, H, hexane, P, paraffin oil)

CAS, Chemical Abstracts Service number

%, minimum purity in percent

through a hole in the glass tube, and blowing an air puff of 2.5 ml during 0.5 sec through the pipette into the air stream, using a stimulus controller (Syntech SFC-1/b). Control stimuli were delivered first, followed by screening stimuli containing multiple compounds (screening blends listed in Table 1). For all screening stimuli that elicited a positive response of approximately≥40 Hz, the pipettes loaded with all compounds in the blend(s) were brought from the

freezer and tested individually after thawing at room temperature for 5 min.

The net response to a stimulus was obtained by counting action potentials (spikes) during 0.5 sec starting from the time after the stimulation period at which the earliest response for the neuron was found, and deducting the number of action potentials during 0.5 sec immediately prior to the response. Each neuron was also subjected to blank stimuli (i.e., only solvent), and the net response to the blank was deducted from the response to the test compounds. The resulting value was doubled to obtain a value corresponding to spikes/sec (Hz). The time between the start of the stimulation period and the onset of a response, i.e., increased number of action potentials, sometimes varied between different recording sessions, due to slight variations in the air flow. For each neuron, counting of action potentials was started from the time at which the earliest response in that neuron occurred.

Field Experiments Related to GC-EAD Field experiments with the sorghum and abutilon compounds were carried out at Rasa, Ethiopia (see above). A complete randomized block design with $N = 10$ was used. The distance between traps was 10 m, and blocks were separated by at least 50 m. Dispensers were placed in cardboard holders (78×37 mm, Silvandersson AB, Knäred, Sweden) fitted into a slot in the vanes of Japanese beetle traps (Trécé, Palo Alto, CA, USA), which were suspended approximately 3 m above ground from wooden poles. The traps were emptied daily, and lures were replaced in the morning before the onset of activity for adult *P. interrupta*. Unbaited traps were used as a negative control.

Experiments were performed during two periods: July 11–16 and October 7–13, 2006. The latter tests were done during the cropping season, when the sorghum had seeds in the milky stage. The July experiments were carried out in a grazing area characterized by scattered *Acacia* trees. In October, traps were placed along the borders of five sorghum fields located approximately 500 m from the July test sites. Compounds were applied to dental cotton rolls (No. 3, Q-dent, Germany). The individual sorghum compounds, (*Z*)-3-hexen-1-ol, tridecane, 1-octen-3-ol, and 1-octanol, were applied at a dose of 100 mg each. In addition to the sorghum compounds, eugenol and methyl salicylate also were tested. Two of the sorghum-related blends were tested both in July and in October: a blend of the four sorghum compounds with the same total dose (100 mg) as for the individual compounds and in a ratio mimicking what was found in the sorghum headspace, i.e., 10 mg (*Z*)-3-hexen-1-ol+30 mg tridecane +30 mg 1-octen-3-ol+30 mg 1-octanol, and the same sorghum blend with the addition of 30 mg methyl salicylate. In addition to these, three more blends were tested in October: the sorghum blend with the addition of 30 mg eugenol, the sorghum blend with the addition of 30 mg eugenol and 30 mg methyl salicylate, and a blend of 50 mg eugenol and 50 mg methyl salicylate.

The individual abutilon compounds were also tested at a dose of 100 mg: (*Z*)-3-hexen-1-ol (the same traps as in the sorghum experiment), tetradecane, methyl anthranilate, and methyl salicylate. Methyl anthranilate was tested only individually in October. An abutilon blend at a total dose of 100 mg and with ratios mimicking the headspace collections was also tested as follows: 20 mg (*Z*)-3-hexen-1-ol+20 mg tetradecane+5 mg methyl anthranilate + 55 mg methyl salicylate. In addition, a blend without methyl salicylate was tested. Furthermore, in October, a blend consisting of the abutilon blend with the addition of 30 mg eugenol was added to the experiment.

Field Experiments Related to SSR The materials and methods used in SSR-related field experiments were the same as those used for field experiments related to GC-EAD, except $N = 5$ and treatments were moved one step within blocks each day to minimize any impact of possible position effects. Some previously untested compounds also were applied to new dispenser types (see below).

Six novel compounds selected by SSR were tested on 4–9 July 2008 on unused farmland with sparse vegetation near the village of Embuay Bad in Ethiopia (09°48′N, 40°00′E), 1206 m above sea level, 265 km northeast of Addis Ababa, Ethiopia. Five of the novel compounds tested (anethole, benzaldehyde, racemic 2,3-butanediol, isoamyl alcohol, and methyl octanoate) were selected on the basis that they elicited strong SSR response in separate ORN classes that did not respond to compounds previously tested in the field. For comparison, we also included eugenol and methyl salicylate. Methyl benzoate was tested for reasons different from the other compounds—it was included since it activated the same ORN type as methyl salicylate, which previously had been shown to be highly attractive. Olfactory receptor neurons responding to eugenol did not respond to other compounds included in the screening process. Practical limitations forced us to forego testing of some compounds as it was not possible to acquire necessary quantities in suitable purity, and other compounds were not included since they are more commonly associated with foliage than fruit or flowers.

A dose of 100 mg of pure compound (for purity and CAS number, see Table 1) was loaded onto a dispenser that was matched to the volatility (as indicated by boiling point) of the compound. This rough estimation was used to obtain comparable evaporation rates. Cotton rolls (no. 2 dental cotton roll, Demedis GmbH, Langen, Germany) were used as dispensers for anethole, eugenol, methyl benzoate, methyl octanoate, and methyl salicylate. For dispensing benzaldehyde and 2,3-butanediol, cotton rolls were pushed into 4 ml

vials (45×14.7 mm, clear, Skandinaviska GeneTec AB, Västra Frölunda, Sweden) until the cotton was level with the rim of the opening of the vial. Compound was applied to the cotton roll after it had been placed in the vial. For isoamyl alcohol, a dispenser was made where a cotton roll was put inside a vial closed with a cap (black, closed top, 13 mm, Skandinaviska GeneTec AB). A hole of approximately 2 mm diam was made in the cap. The cotton roll was placed so that it was in direct contact with the cap when the cap was screwed tight to the vial. The chemical was not applied to the cotton roll directly beneath the hole in the cap, but instead towards the edge of the vial, before screwing on the cap.

Statistical Analysis For field experiments, data for total catch of *P. interrupta* (cumulative over the field testing period) per trap was square root-transformed ($\sqrt{(\times+1)}$). Data was analyzed with a *General Linear Model* (*GLM*), with treatment (type of lure) as a fixed effect, and block as a random effect (Minitab 14 for Windows). Significant *GLM*s were followed by *Tukey's b post hoc* test. The significance level used in all tests was $\alpha=0.05$. Trap catch data is presented in graphs as untransformed means with error bars denoting standard error of the mean.

RESULTS

GC-EAD Analyses and Identification of Host Plant Volatiles Several compounds in both the abutilon (Fig. 1a) and the sorghum (Fig. 1b) headspace collections elicited consistent responses in *P. interrupta* antennae. The EAD-active compounds collected from abutilon headspace extracts were identified as (*Z*)-3-hexen-1-ol, tetradecane, methyl salicylate, and methyl anthranilate (Fig. 1a), and in sorghum as tridecane, (*Z*)-3-hexen-1-ol, 1-octen-3-ol, and 1-octanol (Fig. 1b). Similar antennal responses were recorded from antennae of females (not shown). In abutilon headspace extracts, methyl salicylate was a major constituent, making up approximately 14.4% of the extract. In sorghum extract, however, it was present at low concentrations, and did not elicit any antennal responses. Despite a low concentration of methyl anthranilate in the abutilon extract, this compound elicited a strong antennal response.

Field Experiments Related to GC-EAD During July and October, there were no significant differences in trap catches between the sorghum blend, which caught an average of 12 beetles per trap over the experimental period in July and 11 beetles in October (blend 8, Fig. 2), and the individual sorghum compounds. In July, traps baited with methyl salicylate caught significantly more beetles (21 beetles/trap) than traps baited with tridecane or (*Z*)-3-hexen-1-ol. Eugenol baited traps caught significantly more beetles (40 beetles/trap) than all other treatments, except the sorghum blend with the addition of methyl salicylate (40 beetles/trap, blend 9, Fig. 2a). In October, the sorghum blend with eugenol (82 beetles/trap, blend 10) and the sorghum blend with eugenol and methyl salicylate (70 beetles/trap) attracted by far the highest numbers of beetles, followed by the sorghum

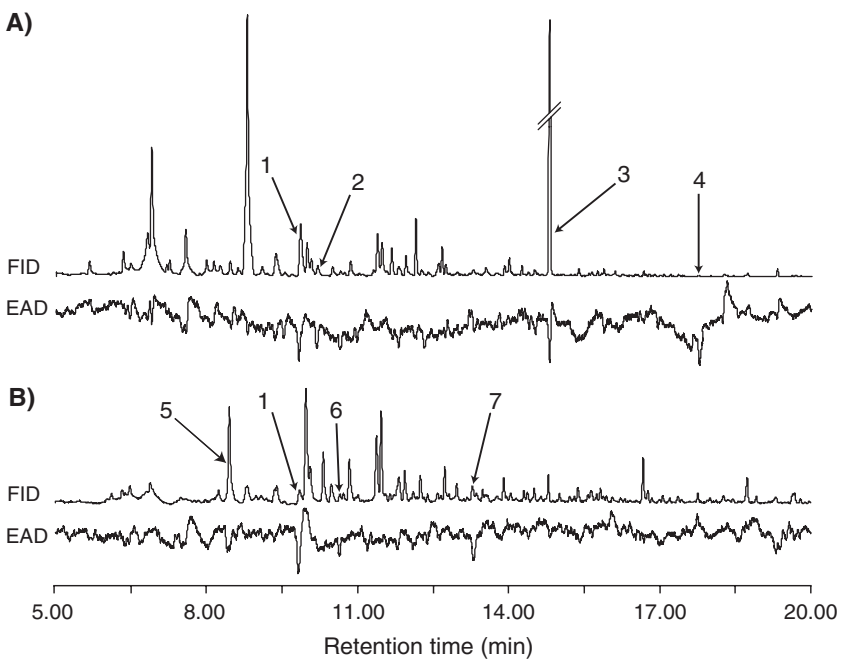

Fig. 1 Simultaneous response of flame ionization detector (FID) and male *Pachnoda interrupta* antennae (EAD) to volatile compounds collected from **a** abutilon and **b** sorghum. The upper traces in each figure represent the signal from the FID and the lower traces represent the signal from the EAD. The compounds collected from abutilon headspace extracts were identified as (*Z*)-3-hexen-1-ol (1), tetradecane (2), methyl salicylate (3), and methyl anthranilate (4), and in sorghum tridecane (5), (*Z*)-3-hexen-1-ol (1), 1-octen-3-ol (6), and 1-octanol (7)

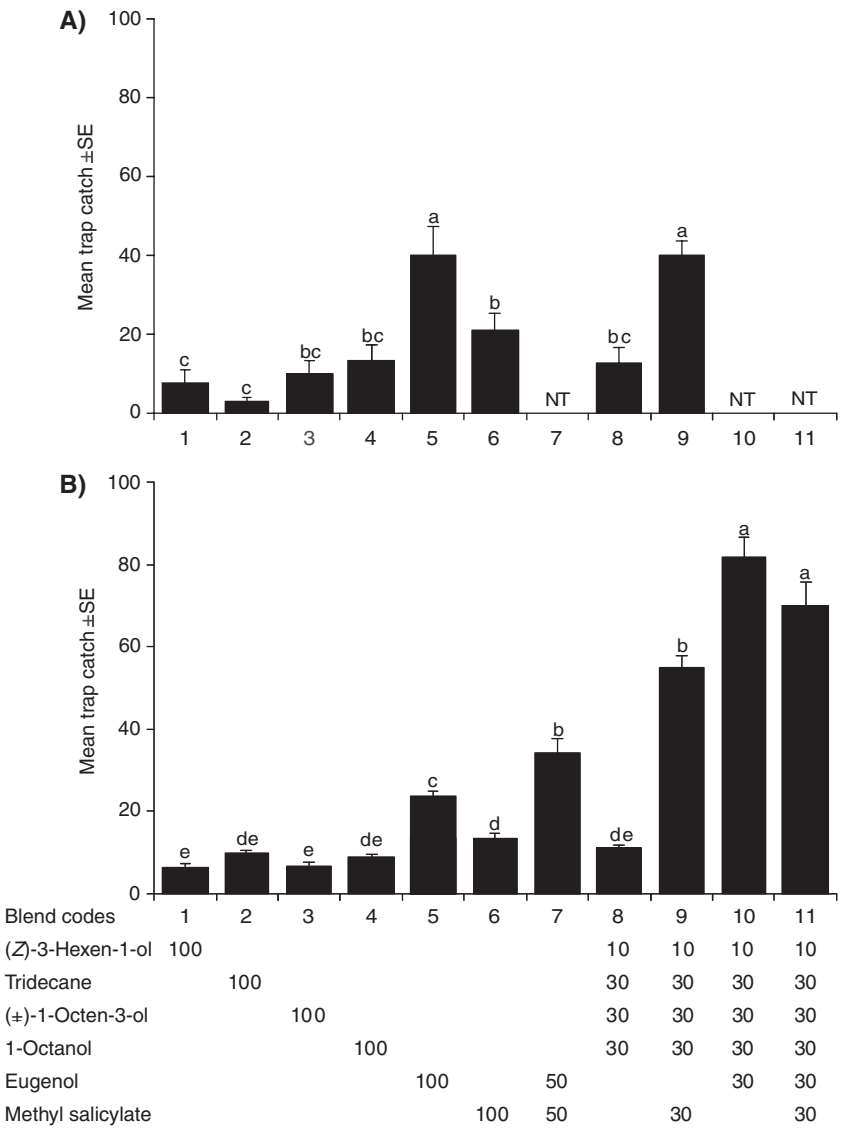

Fig. 2 Number of *Pachnoda interrupta* captured in traps baited with synthetic sorghum compounds in **a** July, 2006 and **b** October, 2006. Unbaited traps caught no insects and were not included in the comparison. *GLM*: July, (N= 10, F=78.1, df=7, 63, P<0.001); October, (N= 10, F=114.1, df=10, 90, P<0.001). Error bars show the standard error of the mean. Subgroups denoted by different letters are significantly different at α=0.05 (N= 10, *Tukey's b*). NT indicates that the treatment was not tested

blend with methyl salicylate, the two-component eugenol-methyl salicylate blend, and eugenol alone. In both July and October, the four-component abutilon blend caught significantly more beetles (22 beetles/trap in July and 17 in October, blend 7, Fig. 3) than traps baited with the single compounds (*Z*)-3-hexen-1-ol and tetradecane. While there were no significant differences in trap catches between traps baited with the abutilon blend and the blend without methyl salicylate (15 beetles/trap) in July, the complete blend caught more beetles in October. In both seasons, there were no significant differences in trap catch between the abutilon blend and methyl salicylate presented as a single compound or eugenol as a single compound. When methyl anthranilate was added as a single compound in October, it was as attractive as the abutilon blend and eugenol and methyl salicylate (20 beetles/trap, Fig. 3b). The other treatment that was added, the four-component abutilon blend combined with eugenol, was equally attractive (42 beetles/trap, blend code 9, Fig. 3b) as the binary mixture of methyl salicylate and eugenol (37 beetles/trap), and more attractive than any other treatment. During both seasons, all treatments caught significantly more beetles than the unbaited control traps. Male and female beetles followed the same pattern of attraction, with no clear differences between the sexes in which baits were most attractive (data not shown).

Single Sensillum Recordings The antennal morphology of *P. interrupta* is similar to that of the closely related scarab *P. marginata*, as described by Stensmyr et al. (2001). *P. interrupta* has a typical scarab antenna, where sensilla are present on the inner surfaces of the three lamellae at the tip of the antenna. Most sensilla are of the placodea morphological type, with a small minority of smooth peg and coeloconic sensilla. Our SSR recordings

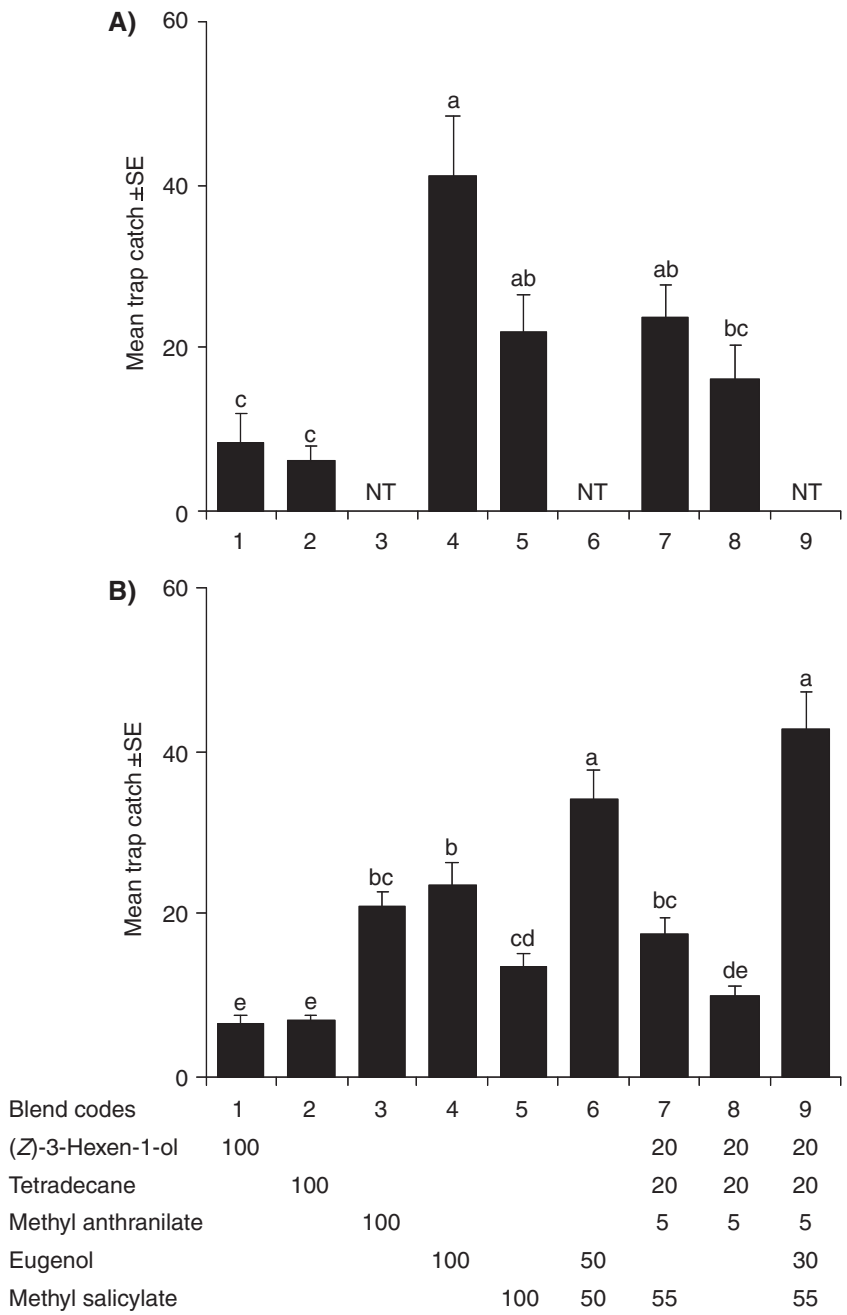

Fig. 3 Number of *Pachnoda interrupta* captured in traps baited with synthetic abutilon compounds in **a** July, 2006 and **b** October, 2006. Unbaited traps caught no insects and were not included in the comparison. *GLM*: July 2006, (N= 10, F=12.7, df=5, 45, P<0.001); October 2006, (N= 10, F=42.1, df=8, 72, P<0.001). Error bars show the standard error of the mean. Subgroups denoted by different letters are significantly different at α=0.05 (N= 10, *Tukey's b*). NT indicates that the treatment was not tested

stem from sensilla placodea, as we only managed to get intermittent contacts with ORNs of the other morphological types.

Contacted sensilla typically contained two ORNs, which in most cases could be separated by differences in spike amplitude (Fig. 4). In sensilla containing two neurons, both neurons sometimes fired simultaneously, resulting in double spikes with amplitudes greater than that of either neuron by itself. When subjected to synthetic stimuli, ORNs usually responded strongly with a train of action potentials (spikes) to a few compounds. Recordings were obtained from a total of 156 sensilla in males and females. Data for the sexes were pooled, as no types of ORNs were numerous enough for a meaningful comparison between the sexes. Out of the 156 sensilla, 50 sensilla contained no ORNs responding to the stimulus spectrum tested. ORN response to stimulation with control stimuli (blank) normally was below 10 Hz (data not shown), but 3 ORNs gave a blank response of 50 Hz or above and were excluded from analysis, since we deemed that there was a risk that the cells had either been injured and were not responding properly, or that some contamination had been present in the odor stimulation system. We also excluded 8 ORNs for which no compound elicited a net response of

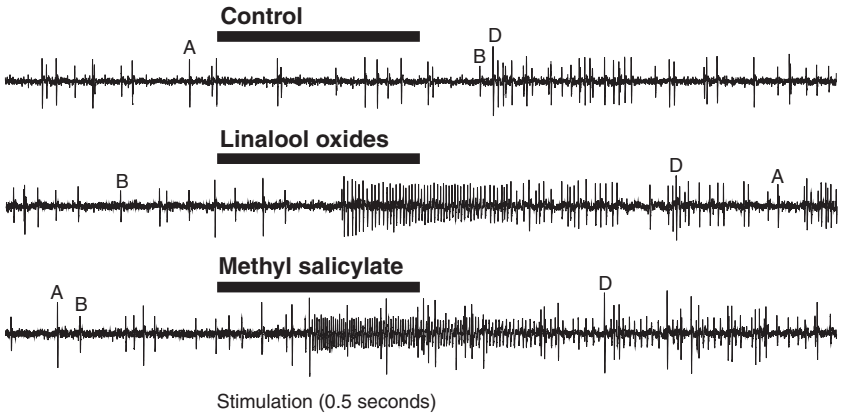

Fig. 4 The two ORNs present in this sensillum are distinguished by the amplitudes of their action potentials. The ORN responding to linalool oxide (denoted "A") has a higher amplitude than the ORN responding to methyl salicylate (B). There are also double spikes, where both neurons fire simultaneously (D), that have a greater amplitude than either neuron by itself

40 Hz or above, as these weak responses were unlikely to indicate key stimuli suited for evaluation in field trials. The remaining 97 neurons responded with a net response of 40 Hz or above to at least one stimulus. Of the 82 test compounds, 57 elicited spike responses over 80 Hz at least once (Table 1). Not all responses could be assigned to a specific class, but 94 responding ORNs could be grouped tentatively into 26 classes (Table 2). In most cases, ORNs responded to a single compound or a group of functionally or structurally similar compounds with one eliciting a stronger response than the rest. For some neurons, several compounds elicited strong responses, with no clear ranking between the 2–3 best compounds. A few ORNs had wide response spectra, with 5–10 compounds eliciting strong responses. For ORN classes, the average response to ligands is shown down to 40 Hz (Table 2).

Several of the ORN classes responded to stimulation with compounds that commonly occur in the volatile profiles of fruit and flowers (Table 2). There also were classes that responded to stimulation with compounds associated with foliage, i.e., green leaf volatiles (GLVs). No ORN response was found to ethanol, acetone, acetic acid, or propionic acid, and there was only one response to ethyl acetate. However, ORNs responded to isovaleric acid, acetoin, racemic 2,3-butanediol, 4-ethylphenol, and 4-methylphenol.

Field Experiments Related to SSR Traps baited with racemic 2,3-butanediol caught significantly more beetles over the experimental period than traps baited with other compounds (an average of 205 beetles/trap, Fig. 5; $F=80.56$, $P<0.001$), catching three times more beetles than the second best bait, eugenol (which caught 69 beetles/trap), and six times more than the third best, methyl salicylate (36 beetles/trap). Apart from 2,3-butanediol, traps baited with previously untested compounds did not catch significantly more *P. interrupta* than the unbaited control traps. Males and females follow the same pattern of attraction; there were no clear differences between the sexes for which baits were most attractive (data not shown).

Discussion

Some insect species require specific blends of several compounds to be attracted to their host (Bruce et al., 2005), e.g., the apple maggot fly, *Rhagoletis pomonella* (Linn et al., 2005), the grapevine moth, *Lobesia botrana* (Tasin et al. 2006, 2007), and the fruit fly, *Drosophila melanogaster* (Zhu et al. 2003; Ruebenbauer et al. 2008). However, for many fruit and flower visiting scarabs, individual compounds constitute efficient attractants (Donaldson et al. 1990). Relying on key compounds rather than specific blends could be an efficient general host detection strategy for a polyphagous herbivore such as *P. interrupta*. Such a strategy could be enhanced by the presence of reliable signals from plants that attract animals for pollination or fruit (seed) dispersal. Our results from field experiments with blends that mimic the hosts abutilon and sorghum indicated that *P. interrupta* is attracted to a few key components in the volatile profiles of these plants, rather than ratio-specific blends (Fig. 2 and 3), and thus we focused our search for attractants on single compounds. As we also noted that the most attractive compounds were associated with fruits and flowers (e.g., eugenol and methyl salicylate), rather than foliage [e.g., (*Z*)-3-hexen-1-ol], we primarily tested floral- and fruit-related kairomones in the single sensillum screening. Among the kairomones selected for screening, many have been found in several hosts of *P. interrupta*, e.g., banana (Macku and Jennings 1987; Ibáñes et al. 1998; Boudhrioua et al. 2003) guava (Carasek and Pawliszyn 2006), mango (Clara et al. 2007; Pandit et al. 2009), and various flowers (Knudsen et al., 2006). We also included fermentation volatiles (Chatonnet et al. 1992; Fischer et al. 2000; Xiao and Ping 2007), since the sorghum

Table 2 Olfactory receptor neuron classes in the sorghum chafer, *Pachnoda interrupta*

ORN class 1: 2-Hexen-1-ol	n = 2
(*E*)-2-Hexen-1-ol [104]	
1-Hexanol [90]	
(*Z*)-3-Hexen-1-ol [88]	
(*E*)-3-Hexen-1-ol [74]	
(*Z*)-3-Hexenyl acetate [47]	

ORN class 2: 3-Hexen-1-ol	n = 6
(*E*)-3-Hexen-1-ol [114]	
(*Z*)-3-Hexen-1-ol [113]	
(*E*)-2-Hexen-1-ol [59]	
(*Z*)-3-Hexenyl acetate [44]	

ORN class 3: Nonanal	n = 7
Nonanal [108]	

ORN class 4: Isovaleric acid	n = 6
Isovaleric acid [85]	

ORN class 5: N-Caproic acid	n = 1
N-Caproic acid [85]	

ORN class 6: Butyric acid	n = 1
Butyric acid [82]	
Valeric acid [60]	

ORN class 7: 4-Methylphenol	n = 5
4-Methylphenol [84]	

ORN class 8: Benzaldehyde	n = 3
Benzaldehyde [72]	

ORN class 9: Benzylalcohol	n = 2
Benzylalcohol [78]	
4-Ethylphenol [46]	
4-Methylphenol [46]	

ORN class 10: Phenylacetaldehyde	n = 2
Phenylacetaldehyde [72]	
Phenylacetonitrile [64]	
2-Phenylethanol [46]	

ORN class 11: Anethole	n = 4
Anethole [110]	

ORN class 12: racemic 2,3-Butanediol	n = 3
racemic 2,3-Butanediol [103]	
(±)-Acetoin [94]	

ORN class 13: Eugenol	n = 3
Eugenol [123]	

ORN class 14: Methyl salicylate	n = 12
Methyl salicylate [110]	
Methyl benzoate [64]	

ORN class 15: Linalool oxides	n = 7
Linalool oxides [86]	
Linalool [70]	

ORN class 16: Geraniol	n = 1
Geraniol [107]	
(-)-*trans*-Citronellol [89]	
(*E*)-2-Hexenyl acetate [85]	
(*Z*)-3-Hexenyl acetate [83]	
Methyl octanoate [82]	
2-Phenethyl propionate [77]	
(*Z*)-3-Hexenyl isobutyrate [75]	

ORN class 17: Geranyl acetate	n = 2
Geranyl acetate [88]	

ORN class 18: (±)-Ethyl 3-hydroxybutyrate	n = 1
(±)-Ethyl 3-hydroxybutyrate [49]	
(-)-*trans*-Citronellol [43]	

ORN class 19: Methyl anthranilate	n = 1
Methyl anthranilate [50]	

ORN class 20: Methyl cinnamate	n = 1
Methyl cinnamate [59]	

ORN class 21: (±)-*beta*-Caryophyllene	n = 6
(±)-*beta*-Caryophyllene [82]	

ORN class 22: Butyl butyrate	n = 6
Butyl butyrate [83]	
(±)-3-Octanol [81]	
Butyl isobutyrate [80]	
Methyl hexanoate [77]	
Isoamyl butanoate [69]	
Isoamyl acetate [65]	
6-Methyl-5-hepten-2-one [65]	
Propyl butyrate [60]	
Ethyl hexanoate [57]	
(±)-1-Octen-3-ol [49]	

ORN class 23: 6-Methyl-5-hepten-2-one	n = 1
6-Methyl-5-hepten-2-one [102]	

ORN class 24: Methyl octanoate	n = 4
Methyl octanoate [101]	
Hexyl acetate [66]	
Methyl hexanoate [56]	

ORN class 25: Isoamyl alcohol	n = 1
Isoamyl alcohol [127]	
(*Z*)-3-Hexenyl isobutyrate [93]	
Methyl octanoate [83]	
Butyl butyrate [67]	
Propyl butyrate [65]	
Linalool oxides [63]	
Ethyl hexanoate [61]	
(*Z*)-3-Hexenyl butyrate [61]	
Linalool [59]	
Methyl benzoate [57]	
Methyl hexanoate [49]	
2-Phenyl ethanol [49]	
Hexyl butyrate [47]	
(±)-Acetoin [47]	
Phenylacetonitrile [45]	
Ethyl butyrate [45]	

ORN class 26: Unsaturated esters	n = 6
(*Z*)-3-Hexenyl isobutyrate [82]	
(*Z*)-3-Hexenyl tiglate [65]	
(*Z*)-3-Hexenyl butyrate [57]	
Geraniol [51]	
2-Phenylethanol [50]	
(*Z*)-3-Hexenyl acetate [42]	

Average net response in Hz to key ligands shown in [brackets]; for n=1, net response shown (all responses above 40 Hz are included)
ORNs grouped according to stimuli that excite their strongest responses
"n" indicates number of ORNs that belong in each class

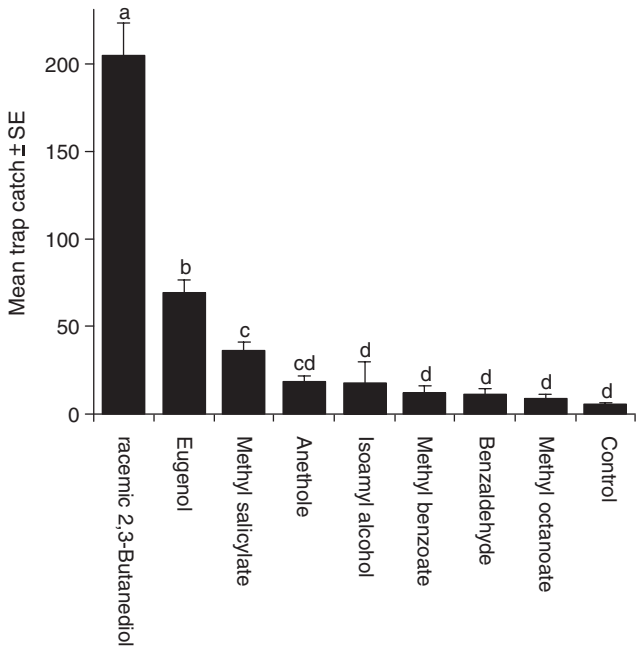

Fig. 5 Average trap catch of *Pachnoda interrupta* in July 2008. The type of bait had a significant effect on catch (*GLM*, $N=10$, $F=80.56$, $df=8, 72$, $P<0.001$). Error bars show the standard error of the mean. Subgroups denoted by different letters are significantly different at $\alpha=0.05$ ($N=10$, *Tukey's b*)

chafer has been reported to be attracted to residue from the fermentation of tella beer. The use of single sensillum recordings (SSR) for screening of potential kairomones enabled us to select compounds for field testing that activate separate olfactory receptor neuron (ORN) classes. Regardless of how information from the olfactory system is interpreted at higher levels of the nervous system, the activation of additional components of the ORN array should increase the likelihood of releasing a behavior, compared to redundant activation of the same ORN classes with several different compounds. Behavioral redundancy between compounds that activate the same ORN class has been observed in the bark beetle *Ips typographus*, where compounds detected by the same ORN do not cause any synergistic repellent effects when combined in field trapping experiments (Andersson et al. 2009). Broad response spectra for ORNs could be an efficient solution for detecting several compounds that relay essentially the same information (Baker et al. 1998; Cossé et al. 1998; Wojtasek et al. 1998; Larsson et al. 2002).

The best field attractants in our study (Fig. 2, 3 and 5) and in a previous study (Wolde-Hawariat et al. 2007) were all detected by single ORN classes — no response to these ligands were found in other ORN classes (Table 2). Thus, activation of a single ORN class seems to be sufficient for the release of attraction behavior. Results from *I. typographus* also indicate that several compounds may cause sufficient activation of a single ORN class to release behavior (Andersson et al. 2009). Methyl benzoate thus was included in the field experiment as it was a secondary ligand of methyl salicylate ORNs (Table 2). Methyl salicylate was highly attractive to *P. interrupta* in the field (Wolde-Hawariat et al. 2007), and to *P. marginata* in a two-choice bioassay (Larsson et al. 2003), even though it was detected by one of the least commonly found ORNs in *P. marginata* (Stensmyr et al. 2001). Methyl salicylate is the primary odorant for a particular ORN in the moth *Mamestra brassicae*, and as in *P. interrupta*, this neuron also has a weaker secondary response to methyl benzoate (Ulland et al. 2008). In *M. brassicae*, egg-laying was inhibited by the presence of methyl salicylate on dispensers near the host plant (cabbage) while methyl benzoate was not tested (ibid.). In our field experiments, catches of *P. interrupta* in traps baited with methyl benzoate were not significantly higher than that of control traps (Fig. 5). Testing of other compounds that elicit secondary responses in ORN classes that respond to attractive compounds could shed light on their role in insect behavior. Despite the fact that *P. interrupta* is attracted strongly to fermentation products such as tella beer residue (Ministry of Agriculture and Ethiopian Agricultural Research Organization 1999), ORNs that respond to primary fermentation products such as ethanol, acetic acid, propionic acid, or acetone, appear to be absent or rare on the *P. interrupta* antenna. Some of these compounds are attractive to other insects that feed on fermenting substrates (e.g., fruit), such as *D. melanogaster*, which are attracted to acetic acid, acetone, and ethanol in lab trapping bioassays (Zhu et al. 2003; Ruebenbauer et al. 2008). We did, however, find ORNs in *P. interrupta* that responded to other substances related to fermentation and microbial degradation, such as racemic 2,3-butanediol, acetoin, 4-ethylphenol, 4-methylphenol, and ethyl acetate (one neuron). The ORN class in *P. interrupta* responding to 2,3-butanediol also responded to acetoin. In the closely related *P. marginata*, acetoin was significantly more attractive than a blank control in a two-choice bioassay, while racemic 2,3-butanediol was not (Larsson et al. 2003). This is interesting as both ligands are detected by the same ORN, and none of the other ORN classes found in the study respond to either compound (Stensmyr et al. 2001). Acetoin and 2,3-butanediol are active in other beetle species as well, where certain isomers or mixtures of isomers are often needed to elicit activity. Meso-2,3-butanediol, (2*R*,3*R*)-2,3-butanediol, and (*R*)-acetoin are emitted by female *Rhizotrogus majalis* and detected only by male antennae in EAD (Nojima et al. 2003). Male *Scapanes australis* emit acetoin, 2-butanol, and 2,3-butanediol, with the first two being necessary and sufficient to attract both sexes in field trapping experiments (Rochat et al. 2002). (*R*)-acetoin has been identified as a female-emitted sex pheromone in the summer chafer, *Amphimallon solstitiale* (Tolasch et al. 2003). In the palm

weevil, *Rhynchophorus palmarum*, acetoin is a synergist to male-emitted volatile aggregation pheromones. The addition of either acetoin or plant matter is necessary to elicit high levels of attraction; the pheromone alone does not suffice (Said et al. 2005).

In the SSR screening, we found ORNs that responded to five of the seven compounds identified as active by GC-EAD (methyl anthranilate, methyl salicylate, (*Z*)-3-hexen-1-ol, 1-octanol, and 1-octen-3-ol), but we did not find any responses to tetradecane or tridecane (Table 1, 2). It is possible that tetradecane and tridecane are detected by one or several rare ORN types, or by ORNs present in smooth peg sensilla or sensilla coeloconica, to which we had only intermittent contacts. Discrepancies between electroantennographic and single sensillum methods have been observed in previous studies (Blight et al. 1995; Barata et al. 2002; Wibe 2004). Wibe (2004) compared GC-EAD and GC-SSR as tools for identification of active compounds for the pine weevil, *Hylobius abietis*, in aerations of sawdust from Norway spruce (*Picea abies*), and found that GC-SSR led to the identification of a higher number of active compounds than GC-EAD.

Apart from racemic 2,3-butanediol, the most attractive compounds in the field were eugenol, methyl anthranilate, and methyl salicylate (Fig. 2, 3, and 5). These compounds are common floral volatiles (Knudsen et al. 2006), with eugenol and methyl salicylate often found in aerations of *Acacia* spp. flowers (Lamarque et al. 1998). Methyl salicylate also is found in leaf odors (Buttery et al. 1982; Loughrin et al. 1997; Shulaev et al. 1997), and is a common defensive compound in higher plants in response to herbivory (Kessler and Baldwin 2001). In field experiments, volatiles emitted from surrounding vegetation thus may include some of the compounds tested, or similar compounds, which could affect the results of field experiments. In the sites used, there is seasonal variation in vegetation and phenology. A salient example is sorghum, which has flowers and seeds in stages attractive to the beetles in October, but not in July. This also coincides with life cycle changes in the beetles, which may affect trap catch. Sorghum chafers mate and feed in July, while in October, the newly emerged adults feed only before going into aestivation until July the following year. Experiments evaluating the effects of these factors were outside the scope of our study.

By using SSR to target a large fraction of the peripheral olfactory system, we identified racemic 2,3-butanediol as an efficient field attractant, without needing to test all of the 57 compounds in our screening that elicited response (Table 1, 2). This compound could be useful in future control or monitoring, especially since it is highly attractive to both male and female *P. interrupta*. Future field experiments should clarify which isomers of 2,3-butanediol (or mixtures thereof) are responsible for attraction. Field tests also should include other fruit- or flower-related compounds that activate so far untested ORN classes, as well as further compounds related to microbial degradation or fermentation, and blends of the best attractants. *P. interrupta* may serve as a useful model for future research on host searching in polyphagous fruit- and flower-feeding insects.

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Canopy gap dynamics have been studied intensively in the cool temperate forests of Japan where *Fagus crenata* is the main climax species (Nakashizuka 1984; Yamamoto 2000). The mortality rate of current-year *F. crenata* seedlings is high because of the occurrence of damping-off at several weeks after germination. Further, the mortality rate is lower for seedlings growing in the gaps than those growing under shaded conditions (Hashizume and Yamamoto 1975; Maeda 1988; Nakashizuka 1988; Sahashi et al. 1994, 1995; Abe et al. 2005). However, the factors that affect resistance to fungal invasion are unclear.

Generally, tree seedlings resist fungal invasion by forming a specific tissue (Tippett and Hill 1984; Eyles et al. 2003; Agrios 2005; Laflamme et al. 2006) and by producing antifungal substances (Bonello and Pearce 1993; Ingham 1973; Siegrist et al. 1994; Agrios 2005; Treutter 2006). Therefore, differences in the resistance of seedlings grown under different light conditions to fungal invasion should be considered by comparing the histological structure (periderm formation) and chemical content (concentration of phenolic compounds) of the seedlings. Increase in the concentration of phenolics has been reported in seedlings grown in gaps or under increased light intensity (Entry et al. 1991; Nichols-Orians 1991). From a phytopathological viewpoint, the confirmation of whether such differences in defense mechanisms are attributable to light conditions in the field is essential for a better understanding of gap dynamics.

In order to determine the resistance factors that affect mortality due to damping-off in current-year *F. crenata* seedlings, we identified the causative fungal species, and we compared the tissue structure of the periderm in the seedling hypocotyls and the change in the total phenol concentrations between current-year seedlings grown under two different light conditions in a natural beech forest in northern Japan.

Methods and Materials

Study Site The field study was conducted in a secondary forest of *F. crenata* (39°59' N, 140°54' E) in Appi, Iwate Prefecture in northern Honshu, Japan. The canopy height in the forest was 25 m. An experimental plot was established along 75 m of the forest road (4 m width, no pavement, and rare traffic) and 25 m into the closed stand. An area less than 5 m from the forest road was designated as the forest-edge plot, and an area more than 10 m wide was designated as the closed-stand plot. The forest-edge plot was established under canopy facing north, and had leaf litter of *F. crenata* that was not extremely dry. The relative illuminations in the closed-stand and forest-edge plots were evaluated by using a light meter, model LI-250 (Li-COR Inc., Lincoln, NE, USA), and they were 2.6% (SE, ±0.22) and 35.5% (SE, ±3.03), respectively.

Seedling Observation, Fungal Isolation, and Inoculation From the forest-edge and the closed-stand plots, 100 and 101 current-year seedlings, respectively, were randomly selected. These seedlings were observed from May 28, 2004 (soon after germination) to September 28, 2004 (just before leaf coloring in autumn) to detect the occurrence of damping-off and to determine the factors related to seedling death. Dead seedlings were collected when their leaves wilted.

Fungus Isolation from Dead Seedlings Seedlings that had died because of damping-off were collected, and the lower parts of their hypocotyls were sterilized in 70% ethanol solution and 1% sodium hypochlorite solution for 1 min each, rinsed twice with sterile distilled water, transferred onto 1% malt-extract agar (MEA), and incubated at 20°C in darkness. Two wk after incubation, the fungi that emerged from the tissues were isolated and maintained on MEA plates. The fungal species to which the isolates belonged were identified on the basis of the morphological characteristics of the cells in the culture medium and on the hypocotyls, as observed under a light microscope.

Inoculation of Isolated Fungi into Seedling Hypocotyls Four fungi, which were isolated at high frequencies from the dead seedlings, were grown on 1% MEA for 3 wk and used for an inoculation test. Seeds of *F. crenata* collected from Appi in October 2005 were peeled, and the cotyledons with the seed coat were sterilized with 1% sodium hypochlorite solution for 1 min. These were then potted in enameled iron dishes (33 cm×38 cm; depth, 5 cm) containing Kanuma pumice (4 cm), which had been autoclaved at 121°C for 30 min. The potted seeds were incubated at 4°C in darkness for 1 mo, according to a previously described method of germination (Katsuta et al. 1998). After germination, these dishes were transferred to a growth chamber (NK system, LP-1.5PH; Nihonika Co., Osaka, Japan), and the seedlings were grown at 15°C under a cycle of 15 hr fluorescent light (3.0 μmol cm^{-2} s^{-1}) and 9 hr darkness for 1 mo until the cotyledons had fully expanded and the first pair of true leaves had developed. Subsequently, several mycelial disks (5 mm diam) taken from the colonies of the four fungal species and non-mycelial 1% MEA disks were inoculated onto a wound sliced into the hypocotyls. We used 19–23 seedlings in each treatment. The development of disease symptoms and the number of dead seedlings were periodically recorded over 20 d.

Inoculation of Pathogenic Fungi under Two Different Illumination Conditions Two pathogenic fungi were used for another inoculation test that was performed under two

different illumination conditions. Seeds were potted in the same dishes as described above and incubated at 4°C in darkness for 1 mo. After incubation, the seedlings were grown in the same growth chamber under either high illumination (36.1 μmol cm^{-2} s^{-1}) or low illumination (2.9 μmol cm^{-2} s^{-1}), achieved by using a shading net, at 15°C under a cycle of 15 hr fluorescent light and 9 hr darkness for 1 mo until the cotyledons were fully expanded and the first pair of true leaves developed. Two pathogenic fungi were inoculated into a wound sliced into the hypocotyls of these seedlings by using the same method as that used in the previous inoculation test. The test seedlings were observed for 2 wk and were categorized as healthy, partially diseased, or wilted.

Tissue Structure of Hypocotyls For histological analysis, 3 healthy seedlings were collected randomly from both the forest-edge and the closed-stand plots on June 1, June 15, July 6, and September 10. The lower parts of the hypocotyls were cut and fixed in 5% glutaraldehyde in 0.1% sodium phosphate buffer, pH 7.2, and stored at 4°C. The parts were rinsed thrice in the phosphate buffer and post-fixed overnight at 4°C in 2% osmium tetroxide prepared in the same buffer. Samples were then rinsed thrice with the buffer, dehydrated in an ethanol series, and embedded in Poly/Bed 812 (Polyscience Inc., Warrington, PA, USA). Thin cross-sections (2 μm) were cut with glass knives on an MT2-B ultramicrotome (Sorvall Instruments; DuPont Co., Wilmington, DE, USA) and gently heat-fixed to glass microscope slides. The sections were stained with 0.1% toluidine blue O for 10 min, destained with distilled water, air dried, mounted in Eukitt mounting reagent (O. Kindler, Breisgau, Germany) beneath a cover slip, and observed under an Optiphoto light microscope (Nikon Co., Tokyo, Japan). In addition, to localize the phenolics in the tissue, an HM440E sliding microtome (Microm, Walldorf, Germany) was used to cut 30 μm-thick cross-sections of fixed hypocotyls of seedlings collected from the forest-edge and closed-stand plots on June 1, June 15, and July 6. The cross-sections were stained with 2% (w/v) $FeSO_4$ solution for about 1 hr to stain polyphenolic compounds with Fe (II) ions (Suzuki et al. 2003). The sections were mounted in the staining solution beneath a cover slip and observed under an Optiphoto light microscope.

Analysis of Total Phenolics Healthy seedlings were collected randomly from the forest-edge and closed-stand plots just after germination on May 28, June 15, July 6, and September 10 in 2004. From the collected seedlings, hypocotyls were taken, and their FWs were measured. Two hypocotyls from 2 seedlings were cut into less than 1-mm pieces with scissors, and the phenolics were extracted in 5 ml methanol in the dark over night at room temperature. The methanol extracts were filtered, adjusted to 5 ml, and stored at −20°C. Total phenolics were analyzed according to the Folin-Ciocalteau method (Waterman and Mole 1994). The methanol extracts (100 or 200 μl) were added to distilled water (900 or 800 μl, respectively). A phenol reagent (500 μl) (Wako, Osaka, Japan) was added to the samples, and samples were mixed by vortex. Na_2CO_3 solution (20% v/v, 2.5 ml) was added continuously and mixed, and distilled water (1 ml) was added to the sample. After 20 min, absorbance of the reactants was recorded at 700 nm (by using an ultraviolet-visible spectrophotometer, V-530; JASCO Co., Tokyo, Japan). A standard curve was prepared as follows: gallic acid (Wako, Osaka, Japan) (0.05, 0.1, 0.2, and 0.4 μg μl^{-1} of methanol) was subjected to the treatment described above for the methanol extracts, and each sample was analyzed thrice; the results were averaged. The amount of total phenolics in the hypocotyls was calculated by using the standard curve. Two seedlings were used for each analysis, and the results of five replicates were averaged. The concentrations of total phenolics were expressed as mg gallic acid equivalents g^{-1} FW (±SE).

Antifungal Activity Tests Seedling hypocotyls were randomly collected from the forest-edge (FW 67.9 g, equivalent to 487 seedlings) and closed-stand (FW 81.2 g, equivalent to 657 seedlings) plots on June 25. They were cut into pieces with scissors into fragments less than 5 mm long and then extracted in methanol (1 l) for 2 d in the dark at room temperature. The methanol extract was evaporated at 35–40°C, and the concentrated sample (approximately 100 ml) was extracted thrice with ethyl acetate (100 ml). The ethyl acetate phase (300 ml) was rinsed twice with saturated NaCl solution (150 ml) and dried overnight with Na_2SO_4. It was then evaporated and used for the antifungal activity test as follows. The ethyl acetate phase, equivalent to FW 5, 10, or 20 mg, was evaporated and dissolved in 50% methanol (5 μl). Samples were added to the wells (10 mm diam) of a microplate (Asahi Techno Glass, Tokyo, Japan) and mixed with the spore solution (2.5×10^3 spores in 500 μl) of a pathogenic fungus. As a control, 50% methanol (5 μl) was used instead of the sample. The plate was incubated in the dark at 20°C for about 48 hr. Twenty-five spores were selected randomly from each well, and the percentage of germinated spores was calculated. Each sample was tested thrice, and the percentage of germinated spores was averaged.

Statistical Analysis The Ekuseru-Toukei 2008 (Social Survey Research Information Co., Ltd.) software package was used for statistical analysis. The difference in survival rate between forest-edge and closed-stand plots was evaluated by Logrank test in survival analysis. Differences in the

amount of total phenolics between the hypocotyls of the seedlings from the forest-edge plot and those of the seedlings from the closed-stand plot were evaluated by Student's *t-test* for each sampling date. The square root of each percentage of spore germination in the antifungal activity test was converted to its arc sine (Sokal and Rohlf 1995), and the differences between the control and the different concentrations of the ethyl acetate phases of the hypocotyls of the seedlings from the forest-edge and closed-stand plots were evaluated by two-way ANOVA and were followed by Tukey's test. Differences were considered significant at $P<0.05$.

Results and Discussion

Seedling Survival and Mortality in the Field Between June and September, seedling death was observed mainly in late June and July after true leaves had expanded fully in both the forest-edge and closed-stand plots. On September 28, the mortality of seedlings was 7% and 50% in the forest-edge and closed-stand plots, respectively (Fig. 1), and was significantly different between the two plots ($\chi^2=42.57$, $df=1$, $P<0.01$, logrank test in survival analysis). All seedlings had died due to damping-off, as indicated by their discolored hypocotyls and wilted leaves; no seedling had been damaged by rodents.

From June to July, the true leaves of most seedlings were damaged by insect herbivory. This damage was classified as severe (complete loss of true leaves and stem above the cotyledons) or slight (partial loss of true leaves). The percentage of severely damaged, slightly damaged, and undamaged seedlings was 18.0, 48.0, and 34.0, respectively, in the forest-edge plot and 2.0, 70.3, and 27.7, respectively, in the closed-stand plot. Damping-off occurred only from the severely damaged seedlings in the forest-edge plot, however, it occurred in every insect-damage category in the closed-stand plot. A part of the severely damaged seedlings survived and produced new shoots from the base of the cotyledons in the forest-edge plot.

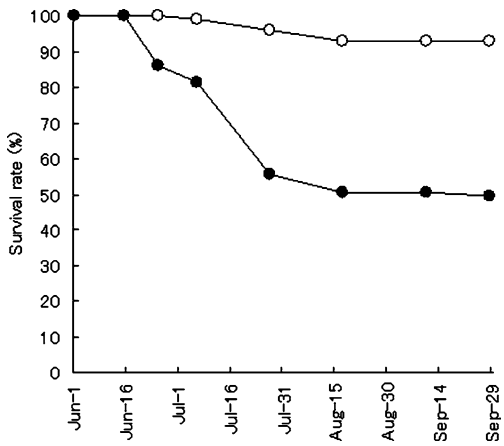

Fig. 1 Survival rates (%) of current-year seedlings grown under two different light conditions in a natural *Fagus crenata* forest. The relative light intensities were 35.5% in forest-edge plot (*open circle*) and 2.6% in closed-stand plot (*closed circle*). *N* seedlings=100 in forest-edge plots and *N*=101 in closed-stand plots

Fungi Isolated from Diseased Seedlings and Inoculation Tests From the lower part of the hypocotyls of the dead seedlings collected from both sites, four major fungi, *Colletotrichum dematium*, *Cylindrocarpon* sp., *Phomopsis* sp., and *Discosia* sp. were isolated (Table 1) throughout the experimental period.

Inoculation Test The results of the inoculation test showed that *C. dematium* and *Cylindrocarpon* sp. induced necrosis in the hypocotyls that resulted in the wilting of the seedlings (Table 2). No disease symptoms were observed in the seedlings inoculated with *Phomopsis* sp. or *Discosia* sp. The results of the inoculation test performed under two different illumination conditions showed that *C. dematium* and *Cylindrocarpon* sp. induced necrosis in the hypocotyls and caused seedling death under low light intensity, but did not induce disease symptoms under high light intensity (Fig. 2).

Periderm Formation Anatomical studies of the lower part of the hypocotyls of the healthy seedlings from each plot revealed histological differences in the epidermal and cortical tissue (Fig. 3). On June 1, live epidermal and cortical tissue was found in the seedlings from both plots (Fig. 3a, e). On June 15, dead epidermal tissue surrounded live cortical tissue in the seedlings from both plots, but the layer of dead epidermal tissue was denser in the seedlings from the forest-edge plot than in those from the closed-stand plot (Fig. 3b, f). On July 6, periderm formation was observed in the seedlings from the forest-edge plot but not in those from the closed-stand plot (Fig. 3c, g). On September 10, periderm formation also was observed in the surviving seedlings from the closed-stand plot, but the periderm was thinner in these seedlings than in those from the forest-edge plot (Fig. 3d, h).

Distribution of Phenolic Compounds Light micrographs of sections of the seedlings from the forest-edge plot immersed in Fe (II) solution showed that epidermal cells were stained black to dark brown on June 1 (Fig. 4a). Positively stained cells were observed in the outer part of the layer of dead epidermal tissue and cortical tissue on June 15 (Fig. 4b) and in the periderm on July 6 (Fig. 4c). The Fe (II)-stained cells were almost always localized with the cells containing black substances in resin-embedded sec-

Table 1 Isolation rate of fungi from hypocotyls of the dead current-year *Fagus crenata* seedlings exhibiting damping-off in two different light conditions, the forest-edge and the closed-stand plots in a natural beech forest

Plot	Isolated fungi	25-Jun	6-Jul	27-Jul	17-Aug	28-Sept
Forest-edge	No. of seedlings	0	9	5	5	0
	Colletotrichum dematium	-	44.4	20.0	0.0	-
	Cylindrocarpon sp.	-	55.6	40.0	20.0	-
	Phomopsis sp.	-	44.4	40.0	80.0	-
	Discosia sp.	-	44.4	80.0	20.0	-
	Other fungi	-	22.2	40.0	100.0	-
Closed-stand	No. of seedlings	7	16	11	3	2
	Colletotrichum dematium	71.4	87.5	54.5	66.7	100.0
	Cylindrocarpon sp.	85.7	62.5	36.4	66.7	100.0
	Phomopsis sp.	0.0	25.0	81.8	33.3	0.0
	Discosia sp.	28.6	18.8	18.2	33.3	0.0
	Other fungi	0.0	6.3	18.2	0.0	0.0

Isolation rate (%) of each fungus was emergence rate from dead seedlings in each sampling date

tions (Fig. 3, 4). The sections of the seedlings from the closed-stand were stained similarly to those from the forest-edge, however, the Fe (II)-stained cells were rare in June 15 and July 6, when the black substance also were rare (Fig 3f, g).

Concentration of Total Phenolics in the Hypocotyls The concentration of total phenolics in the hypocotyls from the forest-edge plot increased after May 28 (Fig. 5), and that in hypocotyls from the closed-stand plot increased slightly after June 15 (Fig. 5). The concentrations in the hypocotyls from the forest-edge plot on June 15 ($P<0.001$), July 6 ($P<0.001$), and September 10 ($P<0.01$) were significantly higher (2.1, 2.2, and 3.7 times, respectively) than the corresponding concentrations in the hypocotyls from the closed-stand plot (Fig. 5).

Antifungal Activity Compared with the control, the ethyl acetate phases of the hypocotyls from the forest-edge plot, equivalent to FW 5, 10, and 20 mg significantly inhibited spore germination of *C. dematium* ($P<0.01$ in each treatment) (Fig. 6). Spore germination also was significantly inhibited by the ethyl acetate phases of the hypocotyls from the closed-stand plot, equivalent to FW 10 and 20 mg ($P<0.01$ in each treatment) (Fig. 6). The inhibitory activity on the spore germination of the hypocotyl extracts from the forest-edge plot was significantly higher than that of the extracts from the closed-stand plot ($F=5.932$, $df=1$, $P<0.05$, Fig. 6). The values of the spore germination were different among the concentration of the ethyl acetate phases ($F=34.46$, $df=3$, $P<0.01$).

In natural *F. crenata* forests in Japan, damping-off in current-year seedlings has been reported to be caused by *C. dematium* (Sahashi et al. 1995). *Cylindrocarpon* sp. also has been isolated from current-year *F. crenata* seedlings that exhibit damping-off (Kobayashi et al. 1984), but its pathogenicity has not been confirmed. We isolated *C. dematium* and *Cylindrocarpon* sp. from diseased hypocotyls of current-year *F. crenata* seedlings that had died of damping-off in both the forest-edge and closed-stand plots (Table 1). We inoculated the four fungal species isolated in this study into the hypocotyls and found that *C. dematium* and *Cylindrocarpon sp.* induced hypocotyls necrosis and damping-off in the current-year seedlings (Table 2). This proved that *C. dematium* and *Cylindrocarpon* sp. are pathogenic to current-year *F. crenata* seedlings, and that damping-off can occur in both the forest-edge and closed-stand plots. The results of another inoculation test con-

Table 2 The percentages of each disease symptom observed on hypocotyls of current-year seedlings under an inoculation test with four fungi isolated from dead *Fagus crenata* seedlings exhibiting damping-off

Inoculated fungi	No. of seedlings	No symptom	Partial necrosis on hypocotyls	Wilted
Colletotrichum dematium	20	70.0	15.0[a]	15.0[a]
Cylindrocarpon sp.	19	31.6	63.2[a]	5.3[a]
Phomopsis sp.	23	100.0	0.0	0.0
Discosia sp.	23	100.0	0.0	0.0
Control	*19*	*100.0*	*0.0*	*0.0*

[a] Inoculated fungi were re-isolated from the diseased seedlings

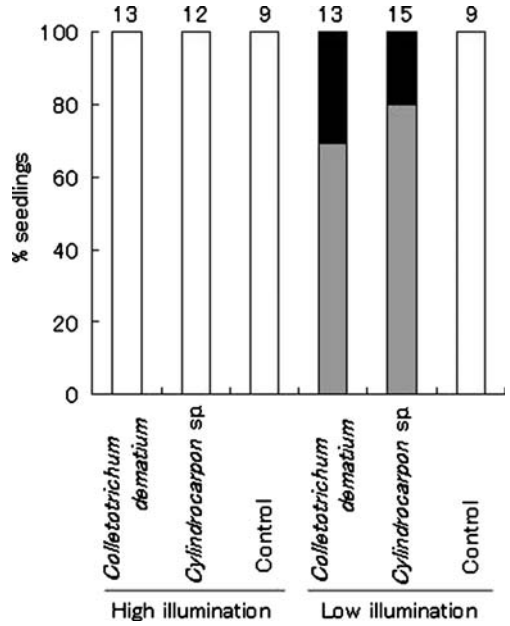

Fig. 2 Current-year *Fagus crenata* seedlings (%) with no disease symptoms (*white bars*), partial necrosis on hypocotyls (*grey bars*), and wilted ones (*black bars*) at different environmental conditions: high and low illumination; after inoculation with the pathogenic fungi, *Colletotrichum dematium* and *Cylindrocarpon* sp. The numbers above each bar indicates the number of seedlings in each treatment (N=100%)

ducted under two different illumination conditions showed that both fungi induced damping-off under low light intensity but not under high intensity (Fig. 2).

Field observations showed that the survival rate of *F. crenata* seedlings was substantially higher in the forest-edge plot than in the closed-stand plot (Fig. 1). Previous field studies also have shown that *F. crenata* seedlings survive better under high illumination, which is observed mainly in gaps, and that under low light conditions, the incidence of damping-off is high (Nakashizuka 1988; Sahashi et al. 1994). Therefore, light deficiency may result in high mortality due to damping-off. We also found from the inoculation test that damping-off occurred under low but not under high light intensity (Fig. 2), thus suggesting that impaired carbon allocation due to low illumination decreases the resistance of hypocotyls to fungal invasion. Therefore, survival of seedlings that grow under the forest-edge may be attributable, at least in part, to an increase in the resistance to damping-off with increased illumination.

In the forest-edge plot, mortality due to damping-off occurred only in the severely damaged seedlings that had been induced by insect herbivory. This suggests that severe leaf loss that impairs carbon allocation decreases the resistance of the seedling hypocotyls to fungal invasion. The survival of a part of the severely damaged seedlings from the forest-edge plot might be attributable to the maintenance of photosynthesis in the cotyledons. However, few insect-damaged seedlings were included in this study, and the relationship between the severity of insect herbivory and seedling mortality needs to be studied by using a greater number of seedlings.

The periderm contributes to the prevention of pathogen invasion in woody plants (Tippett and Hill 1984; Ichihara et al. 2000; Eyles et al. 2003; Agrios 2005; Laflamme et al. 2006). On July 6, we observed that periderm formation in hypocotyls was much more apparent in healthy seedlings

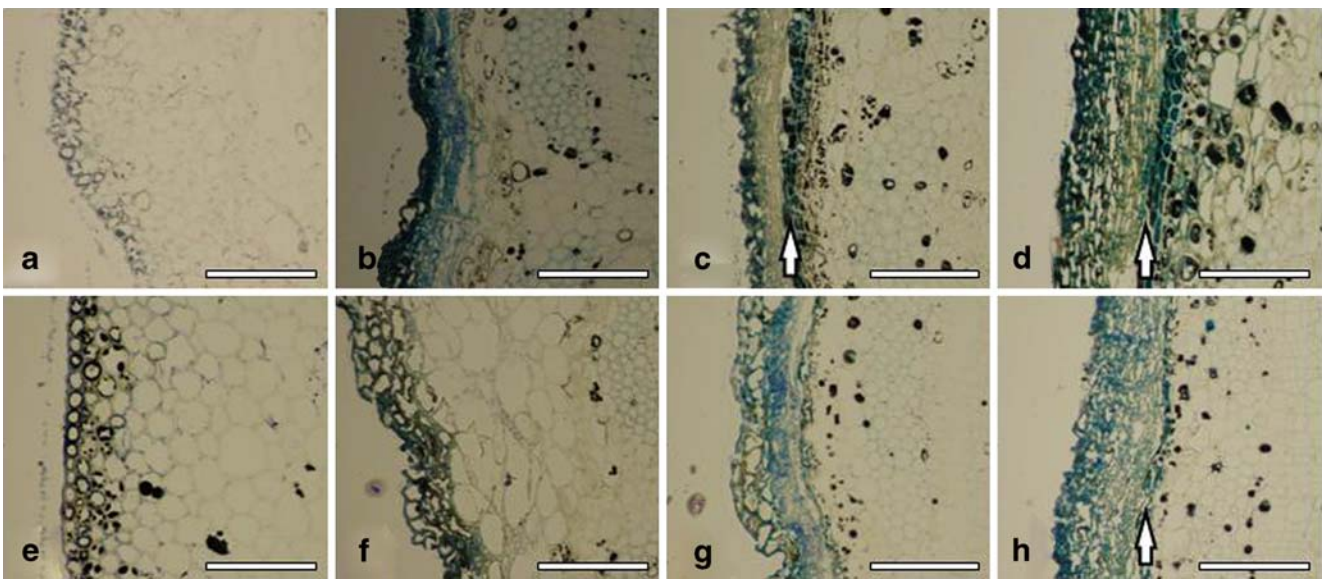

Fig. 3 Microphotographs showing cross sections of hypocotyls of current-year *Fagus crenata* seedlings collected from a natural beech forest. **a-d**: seedlings were collected from the forest-edge plot; **e-h**: seedlings were collected from the closed-stand plot. Seedlings were collected on June 1 **a**, **e**, June 15 **b**, **f**, July 6 **c**, **g** and September 10 **d**, **h**, respectively. Arrows indicate periderm. Scale bars indicate 100 μm

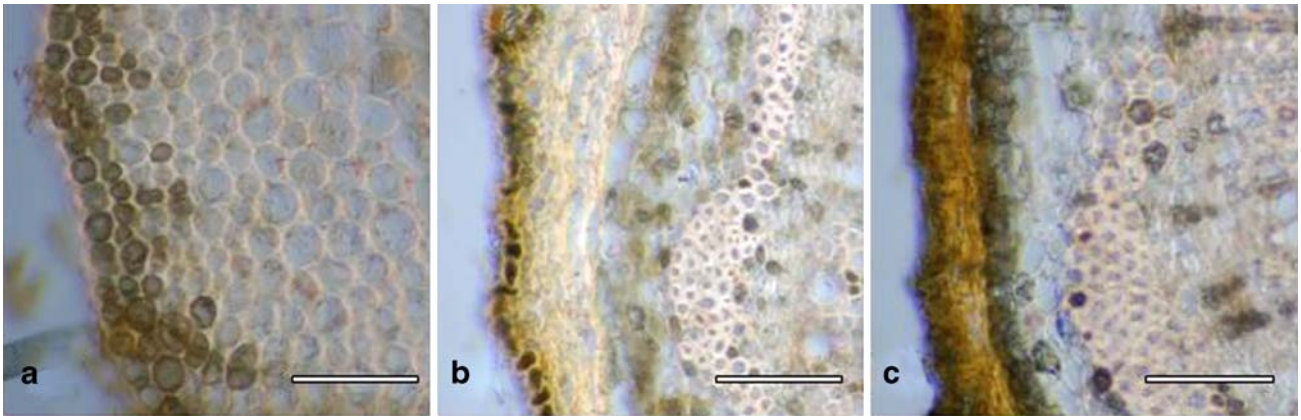

Fig. 4 Microphotographs showing Fe (*II*)-stained cross sections of hypocotyls of current-year *Fagus crenata* seedlings collected from the forest-edge plot in a natural beech forest. The seedlings were collected on June 1 **a**, June 15 **b** and July 6 **c**, respectively. Scale bars indicate 100 μm

from the forest-edge plot than in those from the closed-stand plot (Fig. 3). The periderm may have prevented fungal invasion of the seedlings from the forest-edge plot, which could account for the difference in the mortality between the two plots. Additionally, the layer of dead epidermal tissue, which formed before periderm formation, may also have contributed to the prevention of fungal invasion, because this layer was denser in the seedlings from the forest-edge plot than in those from the closed-stand plot. Periderm degradation and poor development of the dead epidermal tissue layer under low light intensity is likely caused by a decrease in the allocation of photosynthates to hypocotyls because photosynthesis would be decreased in the seedlings from the closed-stand plot. However, the relationship between the allocation of photosynthates and periderm formation in hypocotyls must be studied in more detail.

In general, plants resist fungal invasion by producing phenolic compounds with antifungal activity (Grayer and Harborne 1994; Agrios 2005). Phenolics are involved in the resistance against pathogens in plant seedlings (Bonello and Pearce 1993; Siegrist et al. 1994) and in germinating seeds (Ceballos et al. 1998). The ethyl acetate phase of the methanol extract of seedling hypocotyls from both plots showed antifungal activity, and this activity was higher in the seedlings from the forest-edge plot (Fig. 6). Furthermore, the total phenol concentration on July 6, when damping-off occurred, was higher in the seedlings from the forest-edge plot than in those from the closed-stand plot (Fig. 5). Thus, seedlings in the forest-edge plot may

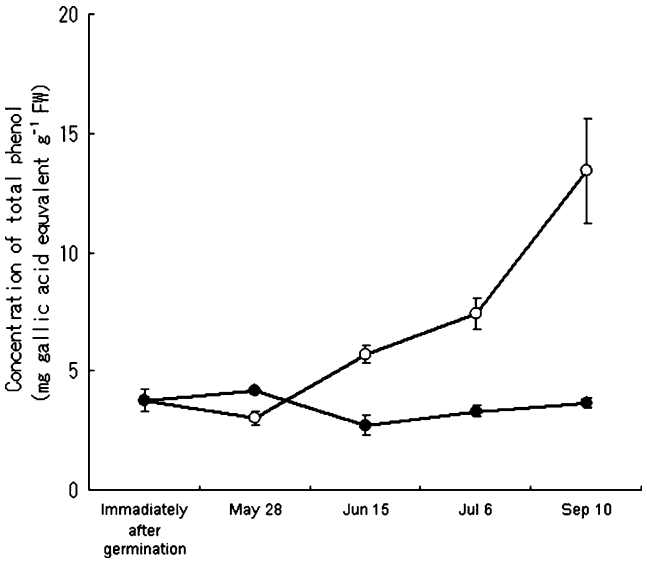

Fig. 5 Concentration of total phenolics in the hypocotyls of current-year *Fagus crenata* seedlings collected from the forest-edge (open circles) and closed-stand (closed circles) plots in a natural beech forest. Data and error bars indicate means and standard errrors (N=5). Hypocotyls were collected immediately after germination on May 28

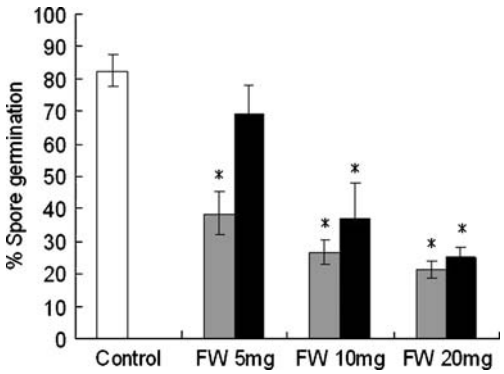

Fig. 6 Antifungal activity of the ethyl acetate phase of the methanol extracts of hypocotyls of seedlings collected from the forest-edge and closed-stand plots in a natural beech forest. The germination of *Colletotrichum dematium* spores with or without the ethyl acetate phases of hypocotyls of the seedlings from the forest-edge and closed-stand plots were observed. The bars and error bars indicate the means of the germination rate and standard errors (N=3). Extracts of seedlings collected from the forest-edge and the closed-stand seedlings are represented with grey and black bars, respectively. Values marked with * are statistically different from the control (Tukey test; P<0.01)

survive by preventing pathogen invasion via phenol production. Additionally, because phenolics were distributed in the outermost parts of the living tissue and in the outer part of the dead epidermal tissue layer, these compounds can effectively impede fungi at invasion. Previous studies have shown that high light intensity results in an increase in the concentration of defense substances, such as total phenolic compounds and tannins (Entry et al. 1991; Nichols-Orians 1991).

We concluded that the difference in the mortality due to damping-off between conditions of high and low light intensity is influenced by both histological and chemical defense mechanisms. The degree of photosynthesis and allocation of photosynthates to hypocotyls could affect the effectiveness of the defense mechanisms against fungal invasion. Increased illumination associated with gap dynamics decreases the incidence of damping-off, which promotes seedling regeneration (Augspurger 1984; Sahashi et al. 1994; Jarosz and Davelos 1995; Hood et al. 2004).

Our results indicate that seedlings growing at improved light conditions associated with gap dynamics show increased resistance against fungal disease. Our study indicates that not only *C. dematium* but also *Cylindrocarpon* sp. are pathogenic and can cause damping-off in current-year *F. crenata* seedlings. Resistance against these fungi could be attributed to changes in production of phenolics and in tissue structure. These changes may be important to promote seedling regeneration. Moreover, because light conditions vary across forest floors, the relationship between light conditions to which each seedling is exposed and the mortality or resistance of the seedling should be studied in association with the resistant factors. This relationship is important for future research in order to understand one of the environmental threshold levels that determine survival of individual seedlings. In addition to light conditions, future studies need to address how other factors such as temperature, humidity, light quality, and host genetic variation affect disease resistance of seedlings. Moreover, the antifungal substances associated with this chemical defense in hypocotyls of *F. crenata* seedlings must be identified.

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The frequent occurrence of PAs in honey (RIVM 2007; Kempf et al. 2008), pollen, and pollen loads of bees (Boppré et al. 2005, 2008) shows that bees are confronted with PAs during natural foraging conditions. Honey foraged from PA-containing plants may contain up to 3.9 µg PAs per gram of honey (Deinzer et al. 1977; Betteridge et al. 2005).

It seems likely that the nectar of PA-plants contains PAs as well, but there is no direct confirmation. On the other hand, there are data on PAs in pollen. These suggest that pollen of PA-plants as well as pollen loads of foraging bees contain high PA concentrations. Up to 14 mg/g for *Echium vulgare* and 0.8 mg/g for *Senecio jacobaea* have been reported (Boppré et al. 2005, 2008). Since floral nectar always is contaminated with plant pollen (which may contain high amounts of PAs) it is difficult to determine the original nectar PA load.

Apart from the ongoing discussion on the ecological role of toxic nectar in plant-pollinator relationships (reviewed by Adler 2000; Raguso 2008), there are numerous examples where plant alkaloids have been shown to be constituents of floral nectars (Baker 1977; Detzel and Wink 1993; Adler 2000; Nicolson and Thornburg 2007). Thus far, deterrence and mortality effects of monocrotaline on butterfly species have been investigated (Masters 1991; Landolt and Lenczewski 1993), but only the effects of a few PAs have been tested against honey bees (Detzel and Wink 1993; Tan et al. 2007). While some work on pollinator toxicity has been performed in the course of pesticide approval, there is only limited information on the mode of action for toxins from natural sources like nectar and pollen. The results of a recent pollen analysis of contaminated honeys (Kempf et al. 2008) indicate strongly that some PA-plants, like *Echium vulgare* are attractive bee plants. To date, there is only one report on the effect of PAs on the fitness and behavior of honey bees: Detzel and Wink (1993) have reported a LD_{50} of 0.1% for the PA heliotrine under no-choice conditions.

The focus of this study was to investigate the effect of PAs in the bee diet on the behavior and health of bees. We were interested particularly to learn how bees handle and metabolize these hazardous plant constituents in order to escape their toxicity.

Since honey bees are social insects that live in colonies of up to 80,000 individuals, we chose laboratory experiments with small caged bee groups. These tests were designed in accordance to the OECD guidelines for the testing of chemicals (OECD 1998: Honeybees, Acute Toxicity Test).

Since only 1,2-unsaturated tertiary PAs but not their *N*-oxides are converted into toxic intermediates through bioactivation by cytochrome P450 enzymes, 1,2-unsaturated tertiary PAs are regarded as pro-toxic, while the respective PA-N-oxides (PA-Noxs) are non-toxic *per se*. However, the *N*-oxides are reduced easily to the pro-toxic free bases in the presence of weak reducing agents. Generally, this happens in the intestines of any herbivore that feeds on a PA-plant. This is why PA-containing plants are essentially toxic although they often store PAs exclusively as *N*-oxides in their tissues (Hartmann and Witte 1995) (Fig. 1).

1,2-Unsaturated PA toxification by hepatic cytochrome-P450 enzymes is well-studied in vertebrates, and acute, chronic, and genotoxic effects are documented (Culvenor et al. 1976; Mattocks 1986; Stegelmeier et al. 1999; Fu et al. 2004). The bioactivation is initiated by hydroxylation of the necine base followed by a spontaneous dehydratization to form pyrrolic ester structures that readily react with cellular nucleophiles (Fu et al. 2004). The structural prerequisite here is the concurrent presence of the 1,2-double bond and an allylic ester functionality. Similar mechanisms can be assumed for mutagenic and toxic effects observed in insects (Frei et al. 1992; Narberhaus et al. 2005; Hartmann and Ober 2008).

Hence, the tests should shed light on the question whether there are different impacts on bees for tertiary PAs and PA-Noxs, respectively. These studies were complemented by feeding monocrotaline and 1,2-dihydro-monocrotaline. These two compounds are structurally closely related, but the latter does not bear the generally required cytotoxic feature of the 1,2-double bond. By studying these compounds, it should be possible to link an observed mortality directly to the toxic principle of the 1,2-double bond.

We also tested known steps of PA metabolism in insects to see whether bees have evolved mechanisms to cope with

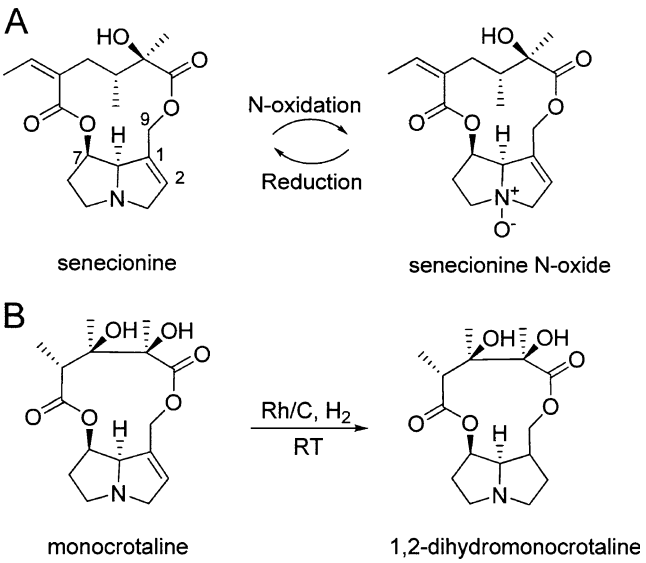

Fig. 1 Examples of pyrrolizidine alkaloid (PA) structures and reactions

natural occurring PAs. In these studies, we intended to monitor elevated excretion of ingested PAs, detoxification of ingested tertiary PAs via *N*-oxidation, and toxification via reduction of 1,2-unsaturated PA-Noxs into 1,2-unsaturated tertiary PAs.

Finally, we addressed the question to what extent trophallaxis is influenced by PA-food uptake. Inside bee colonies, a pronounced horizontal nectar transfer from bee to bee can be observed. Furthermore, foraging bees that return to the hive with poor-quality food experience difficulties in finding unloader bees that are in charge of unloading and storing the nectar in the hive. This in turn results in lower recruiting success from dance attendees to head for this poor food source. Overall, high PA content (in our sense i.e., poor quality) could trigger a lower PA intake into the hive if there were deterrence effects because of high PA concentrations.

Methods and Material

Plant Material Plants of *Senecio vernalis* were obtained in spring 2007 from a wild population in the vicinity of Braunschweig. The inflorescences were collected, lyophilized, powdered, and stored dry in the dark at room temperature until alkaloid extraction.

Bees Summer bees (*Apis mellifera*) collected from a honeycomb from the edge of the colony were used for the feeding experiments. Immediately after the tests, bees where anaesthetized by gaseous CO_2, stored at −18°C, and lyophilized just before analysis.

Preparation of the PA-mix Lyophilized plant material (*Senecio vernalis*, flowering specimens) was extracted according to Hartmann and Toppel (1987). The purified mixture of total plant PAs was analyzed by GC-FID/NPD and GC-MS. It contained senecionine (81%), seneciphylline (15%), integerrimine (1.5%), retrorsine (0.9%), and senecivernine (0.4%). No other components were detected by this method. This purified mixture of tertiary PA is referred to as "PA-mix".

Chemical N-oxidation of Tertiary Alkaloids The *N*-oxidation of the PA-mix from *S. vernalis* (see above) was performed as reported by Cymerman Craig and Purushothaman (1970). All alkaloids of the PA-mix were converted chemically to the corresponding PA-Nox (Fig. 1, *A*) with essentially the same quantitative composition as the "PA-mix". This mixture is referred to as "PA-Nox-mix".

Preparation of 1,2-Dihydromonocrotaline Monocrotaline (99.0%) was obtained from Fluka (Steinheim, Germany). Hydrogenation of the 1,2-double bond was performed as recently described by Marín-Loaiza et al. (2008). Further purification was achieved by column chromatography on silica gel 60 (mesh 230–400) using the solvent system CH_2Cl_2/MeOH/NH_4OH_{conc} (83:15:2/ v:v:v). The purity of the collected fractions was checked by capillary GC and GC-MS analysis. Fractions that contained only 1,2-dihydromonocrotaline were combined. Reaction products of several reactions were combined yielding about 100 mg (average yield per reaction: 40%). Purity and identity were confirmed by GC-MS.

Feeding Experiments Feeding experiments were conducted in the summer seasons 2007 and 2008 with two different bee colonies. Because of the relative early onset of fall conditions in summer 2007, it was not possible to finish all tests in time. To assure as much as possible the highest possible degree of reproducibility in the data set, we here refer to results obtained in 2008. Where possible, we complemented and confirmed the results with data from 2007. The experiments were conducted with minor modifications to the recommended OECD Guidelines for the testing of chemicals (1998). The OECD guidelines recommend one test series with 3 cages that contain ten bees for each concentration (30 bees per data point). In order to generate a more solid dataset, we conducted 3 independent test series for each compound and each concentration (equals 90 individual bees per data point). The particular test compounds (PA-mix, PA-Nox-mix, monocrotaline, and 1,2-dihydromonocrotaline) were dissolved in a 50% (*w*/*w*) sucrose solution at concentrations of 0.02, 0.2, and 2.0% (*w*/*w*). In the 2007 experiments, in addition to these concentrations, a PA level of 0.002% was tested, but omitted for subsequent experiments because it did not display any effects.

In a preliminary experiment, PA-containing diets were offered to bees, and their effects on the feeding behavior and mortality were studied. The PA diets consisted of a purified mixture of 1,2-unsaturated PA isolated from a natural source (*Senecio vernalis*). Two diets were offered: A mixture of the tertiary PAs (free bases) and a mixture of the respective *N*-oxides (Nox). A 0.2% PA content is what one can expect for flower heads of PA-plants. Furthermore, the highest reported value for the PA content in pollen is 1.4% (Boppré et al. 2005). For floral nectar, unfortunately, no data are available. The tested concentrations ranged from 0.02 to 2%, and covered the maximum PA level that an individual honey bee might encounter in nature.

The second test series (monocrotaline/1,2-dihydromonocrotaline) was conducted to examine two questions: (1) Are observed effects of the PA-mixes really caused by the PAs and not by contaminations that possibly were not removed completely during PA-mix purification? (2) Since

the 1,2-double bond is a prerequisite for PA toxicity, what is the effect of the structurally related 1,2-saturated derivative? Monocrotaline was chosen because it is readily available and 1,2-dihydromonocrotaline because it can be obtained with high purity through chemical synthesis. Monocrotaline and 1,2-dihydromonocrotaline were not used to test for differences between 11- and 12-membered PA ring systems.

All solutions were offered to starved bees for 2 h. Starvation status was checked approximately 2 h after the bees were separated from the colony by dissecting 10 randomly picked bees and checking the content of the honey stomach. If all honey stomachs were confirmed empty, the tests were started. After 2 h, the food was changed to a pure 50% sucrose solution *ad libitum* for an additional 46 h. The amount of the consumed PA solution was determined as the loss of weight of the feeding dish after the first 2 h feeding period. Together with each PA test series, two control groups were conducted. One control group (required for the OECD protocol) obtained pure sucrose solution during the first 2 h, while the second did not get any food during the first 2 h. Since all bees were already starving before the tests started, this adds additional stress (starvation) that might also cause mortality. A high mortality in the first 2 h, however, would be reflected in lower food consumption, which then would be misinterpreted as deterrence. The additional control was introduced to recognize deterrence and to distinguish whether mortality in the PA feeding test is a consequence of additional starvation stress induced by strong deterrence or the result of the PA content of the diet. All cages were kept in an incubator at 25±2°C, in darkness, and at a relative humidity of 50–70%. Dead bees of each cage were collected, and the numbers of dead bees were counted after 2, 4, 6, 24, 30, and 48 h. The dead individuals of each time point were stored separately, so subsequent chemical analyses could be done in consideration of the time of death.

Alkaloid Extraction from Bees Lyophilized bees were ground with stainless steel balls (1 to 4 mm diam) in a paint shaker (Merris Minimax MK4, Glattbrugg, Switzerland) in two cycles of 90 s each. Extraction of the bees was conducted according to Hartmann and Toppel (1987). Briefly, homogenates were extracted twice with 500 µL 1 M HCl and left to stand for 10 min. Subsequently, extracts were centrifuged (10 min at 13,000 rpm) and subjected to one of the following work-up methods:

Method A: This was used for monocrotaline and 1,2-dihydromonocrotaline feeding solutions and the horizontal transfer experiment. The extract was made basic with NH_4OH (25%) and applied to an Extrelut® NT20 column (Merck, Darmstadt, Germany) (1.4 ml/g Extrelut®). The PAs were eluted with CH_2Cl_2 (6 ml/ g Extrelut®). The organic solvent was evaporated, and the residue was dissolved in 50 µl MeOH. The amount of monocrotaline, 1,2-dihydromonocrotaline or tertiary PA-mix per bee was quantified by using heliotrine as an external standard (concentration 1 mg/ml).

Method B: This was used for analysis of extracts that contained exclusively PA-mix. Immediately after grinding of the bees, 50 µl of a monocrotaline solution (1 mg/ml in MeOH) were added as internal standard. Monocrotaline was used instead of heliotrine for quantification, because of chromtographically insufficient peak separation for peaks of interest. The procedure was subsequently processed as described for method A.

Method C: This was used for the determination of tertiary PAs and PA-Noxs. To evaluate the ratio of tertiary PAs vs. PA-Noxs, essentially method B was applied, but the supernatant obtained after centrifugation was divided into halves. One half was treated as described in method B to account for the amount of tertiary PAs. To the second half, an excess of Zn dust was added to reduce the PA-Noxs into the corresponding tertiary PAs (see also Fig. 1, *A*). After stirring at room temperature for 3 h, the solution was made basic, and the extraction was finished as described in method A. The result accounted for the sum of tertiary PAs and PA-Noxs (total PAs). The content of PA-Noxs was calculated by subtraction of total PAs by tertiary PAs.

The total PA uptake was calculated on the basis of food consumption during the 2 h feeding period, and correlated to the recovered PA amount.

Analytical Procedures GC-FID/NPD analysis: An Agilent 6890N Network GC System (Agilent Technologies, Wilmington, DE, USA) was equipped with a ZB-1 capillary column (30 m×0.32 mm×0.25 µm f_{th}, Phenomenex, Aschaffenburg, Germany). Conditions: injector 250°C; temperature program 100°C (3 min)–6°C min–310°C (3 min); split ratio 1:10; injection volume 1 µl; carrier gas He (1.2 ml/min). The eluting compounds were detected simultaneously by using a fused-silica Y-splitter and an FID and an NPD detector.

GC-MS analysis: An Agilent 6890N Network GC System was coupled with an Agilent mass spectrometer 5975B inert EI/CI MSD. For GC separation, a DB-1 fused-

silica column (30 m×0.32 mm×0.25 µm f_{th}, J&W Scientific, Waldbronn, Germany) was used. Conditions were the same as above.

The quantitative analysis of the PAs was performed by capillary GC-FID, the identification of the individual PA structure was confirmed by GC-MS in comparison to spectra and retention indices (RIs) recorded from authentic reference compounds (Witte et al. 1993). The retention index (RI) was calculated by a set of hydrocarbons (even numbered from C10 to C28) by linear interpolation.

Horizontal PA Transfer (Trophallaxis) The experiment of bee-to-bee transfer of PAs was performed in the summer season 2007. Recent studies have shown that PAs frequently are found in retail honey and in pollen loads collected by bees (RIVM 2007; Boppré et al. 2008; Kempf et al. 2008). We wanted to test whether the uptake of PA-food influences the exchange of PA-contaminated food among bees. These experiments were planned as a simulation of the natural occurring plant-to-bee and bee-to-bee transfer, and the final PA-deposition in the honey comb.

Two cages with 10 bees each were supplied with a diet containing 0.2% or 2% PA-mix in 50% sucrose solution for 1 h. After 1 h the PA diet was removed and an additional ten bees were released into the cages. These bees were marked with yellow color spots and had no food access beforehand. After 1 h of contact (without any food supply) the experiment was stopped, and the two groups (marked/not marked) were analyzed separately for PAs. The experiment was repeated three times. PA extraction and PA content was determined as described above. All bees of the experiment were analyzed per group (N=10 individuals per group) and thus the values represent the mean PA content per group.

Statistical Methods Means of food consumptions and mortalities in the feeding experiments with PA-mix, PA-Nox-mix (Fig. 2), monocrotaline, 1,2-dihydromonocrotaline (Fig. 4), and the total tertiary PA amounts in the different bee-groups of the feeding experiment with 2%-PA-mix (Fig. 5) were tested for significant differences by One-way ANOVA followed by a Student-Newman-Keuls test for all pairwise comparisons. These tests were performed by using SigmaStat® 3.1 (Systat Software GmbH, Erkrath, Germany).

Results

PA-mix and PA-Nox-mix Feeding Experiments The feeding of the PA-Nox-mix showed a significantly lower food consumption at the highest concentration tested (2% PA content, Fig. 2), indicating substantial deterrent effects. Concerning mortality, only the 2% PA-mix (containing 1,2-unsaturated tertiary PA) showed a marked increase (Fig. 2), while the PA-Nox-mix did not cause any mortality.

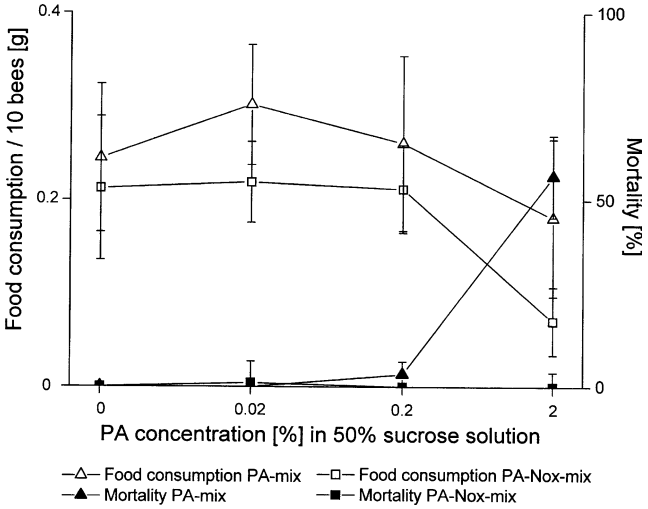

Fig. 2 Comparison of food consumption and mortality (means ± SD) in experiments (N=9) with tertiary PA-mix and PA-Nox-mix. The food consumption of 2% PA-Nox-mix was significantly different (P<0.01) to all other concentrations. The same applies for the mortality observed for 2% tertiary PA-mix (significant difference (P<0.01) to all other concentrations)

In parallel to the regular control groups (0% PA) that had access to sucrose solution throughout the test (48 h), a second control group was established. The time-depending mortality of the starving control group was compared to the 2% PA-mix group and the 0% PA control group (Fig. 3). Figure 3 shows that there can be a high mortality of up to 45% caused obviously by starvation within the first 2 h. But as soon as the remaining individuals had access to regular food (after 2 h), they recovered immediately, and fatal casualties were no longer observed. On the other hand, the increase of mortality caused by the 2% PA-mix clearly showed a different time course. Up to 6 h the mortality rate increased slowly then escalated and kept on ascending and reached mortality rates up to 70% after 48 h (Fig. 3). As expected, the 0% PA control group showed almost no mortality. All individual test series exhibited the same tendencies.

Because stress related mortality induced by starvation could be excluded, the observed mortality rate in PA feeding experiments must be linked directly to PA toxicity and not to PA deterrence and rejection of PA food.

Monocrotaline and 1,2-Dihydromonocrotaline Feeding Experiments To expand the results for the PA-mix experiments, a second test series was conducted. Instead of the PA-mix, pure monocrotaline and its 1,2-dihydro derivative (Fig. 1, *B*) were applied. The feeding protocol was exactly

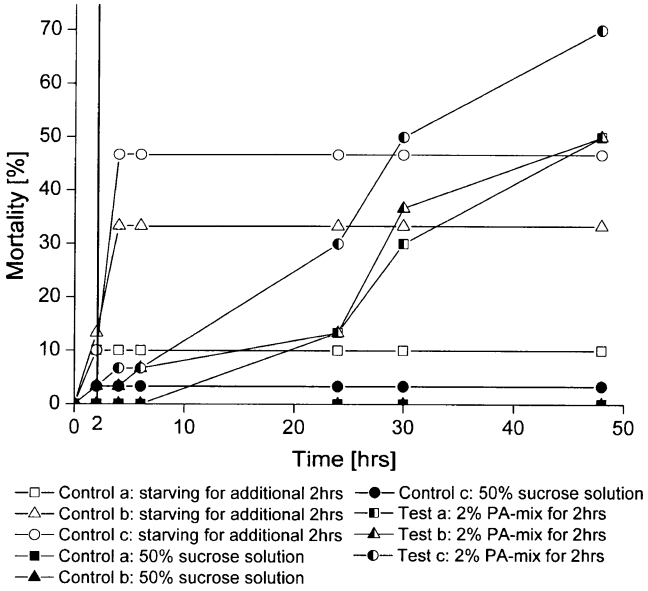

Fig. 3 Comparison of bee mortality in experiments with 2% PA-mix diet (offered for 2 h) (half filled symbols) and the control experiment starvation for 2 h (empty symbols) respective 0% PA diet (*filled symbols*). The curves a to c represent three individual test series (N=10). The time point of food replacement into 50% sucrose solution *ad libitum* is marked (2 h)

the same as that used for the PA-mix experiments. No significant deterrence was observed for these compounds (Fig. 4). Monocrotaline caused mortality effects at concentrations of 2%. The curve progression of monocrotaline resembled the result obtained for the PA-mix. On the other hand, increasing concentrations of 1,2-dihydromonocrotaline did not cause a marked increase in mortality (Fig. 4). The analysis of the control groups showed the same pattern

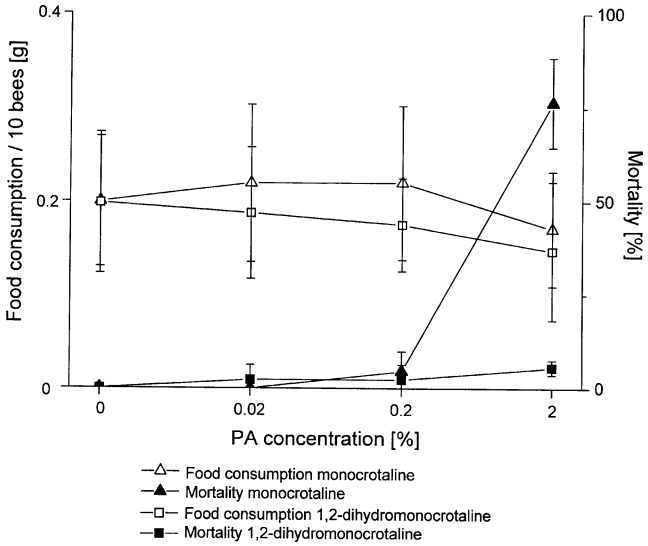

Fig. 4 Comparison of food consumption and mortality (means ± SD) in experiments (N=9) with monocrotaline and 1,2-dihydromonocrotaline. The mortality of 2% monocrotaline was significantly different (P<0.01) from all other concentrations

as obtained for the PA-mix (Fig. 3). The mortality profile for these tested compounds established that the 1,2-double bond of the PA nucleus is an essential feature of PA toxicity in honey bees.

Chemical Analysis of PA-Treated Bees Qualitative and quantitative PA analysis was conducted for distinct groups of bees. The observed effects of the PA feeding should be correlated to the total PA content (PA and PA-Nox) found in individuals of different experiments. The bees of the respective group were picked randomly and analyzed individually. We distinguished early deceased (2 to 30 h), late deceased (>30 h), and surviving bees. The results are summarized in Fig. 5. Bees of the first group showed on average 175 µg PA/bee. The second group exhibited an average PA content of 250 µg/bee. The surviving individuals (48 h) had the lowest PA load (in average 50 µg PA/bee). Because of the selected experimental conditions (sucrose feeding, darkness, humidity, and temperature) bees did not defecate during the assays (48 h). Thus, the PA levels of bees after the treatments were not altered by excretion but only by metabolism.

The comparison of the PA-profile of the PA-mix offered with the diet and the profile of PAs found in bees after 48 h (deceased and surviving bees) demonstrated no significant alteration (Fig. 6). This suggests no discrimination in the uptake of individual PAs from the mixture, and identical metabolic stability of the compounds stored in the bees' bodies. To check for an overall recovery of administered PAs, all bees of one cage were analyzed quantitatively. About 61% of the consumed PAs could be extracted and analyzed by GC-FID/NPD and GC-MS methods. For a single bee, analyzed 46 h after feeding on the PA diet, a maximal load of 200 µg was calculated (Fig. 5, *C*). Considering the average body weight of 100 mg, this amount of 0.2% PAs testifies to a relatively high tolerance towards PAs under these conditions. However, there is an enormous variation of almost one order of magnitude between the PA loads of the individual bees within one experimental set (Fig. 5, *B*).

Conversion of PA-Noxs to Tertiary PAs In most PA-containing plants, bees are facing the non-toxic PA-Noxs, which are the dominating plant constituents. As already mentioned, these *N*-oxides are easily reduced in the intestines of herbivores, yielding the pro-toxic tertiary PAs. Obviously, bees were not harmed by PA-Nox feeding (see Fig. 2). Hence, we wanted to check whether bees have developed mechanisms to maintain the PAs in the non-toxic *N*-oxide state. Individual bees of a group that had access to a PA-Nox-mix (2%) for 2 h and subsequently 46 h of regular food were analyzed for co-occurrence of both PA-forms (Method C). The result indicated that some reduction

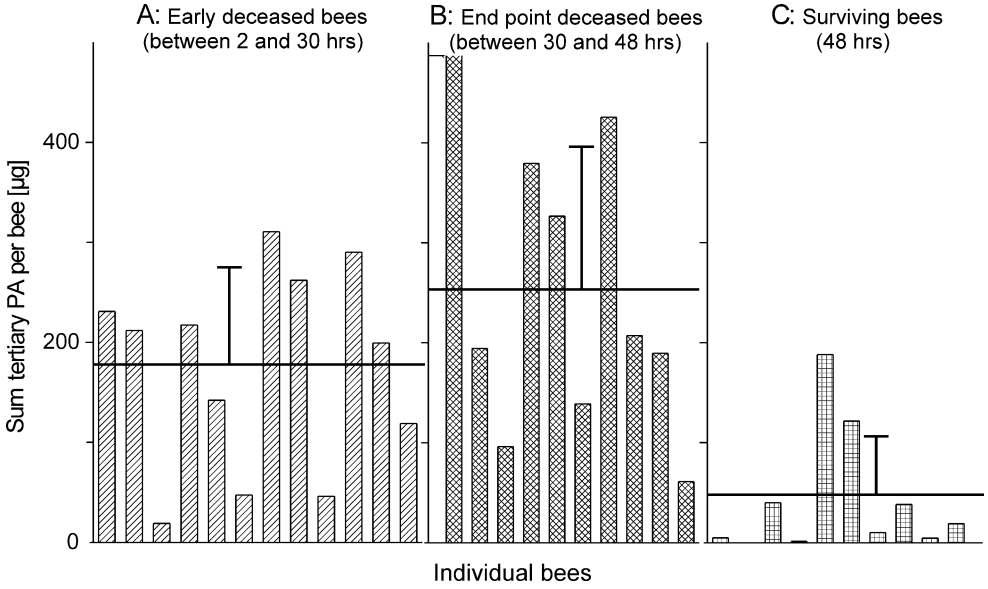

Fig. 5 Amount of pyrrolizidine alkaloid (PA) per individual bee (means ± SD) after feeding of 2% PA-mix divided into different groups: Early deceased bees (between 2 and 30 h) (*A*); end point deceased bees (between 30 and 48 h) (*B*); surviving bees (48 h) (*C*). Total tertiary PA amounts in bees of group *C* were significantly different ($P<0.05$) from total tertiary PA amounts found in group *A* and *B*

occurs. An average of 69% of the administered PA-Noxs was converted into tertiary PAs (Fig. 7). Thus bees have no specific mechanism to maintain the N-oxides in the harmless form. The maximum level of tertiary PAs did not exceed 50 µg/bee (Fig. 7, bee #8). The overall amount of PAs seems rather low, but this is most likely caused by the strong deterrence effect of the PA-Nox-mix (see Fig. 2).

Conversion of Tertiary PAs into PA-Noxs As mentioned above, some specialized insects are able to convert potentially hazardous tertiary PAs into PA-Noxs for "safe" storage or fast excretion. We analyzed bees that had access to the tertiary PA-mix (2%) for 2 h and subsequently 46 h of regular food for the occurrence of possible PA-Nox by using Method C. In all analyzed bees, no such conversion was detectable.

Horizontal Bee-to-bee PA Transfer The bees without direct PA contact showed approximately 4% (2% PA diet) and 15% (0.2% PA diet) of the PA load that was found in bees that had direct PA contact. Thus, at least under laboratory conditions, we demonstrated that horizontal transfer (trophallaxis) of PA contaminated food is possible. In addition, if the content of the honey stomach contained high PA concentrations, the observed exchange to other bees was reduced.

Discussion

Recent studies have documented PA contamination of honey, and have raised concerns of potential health risks

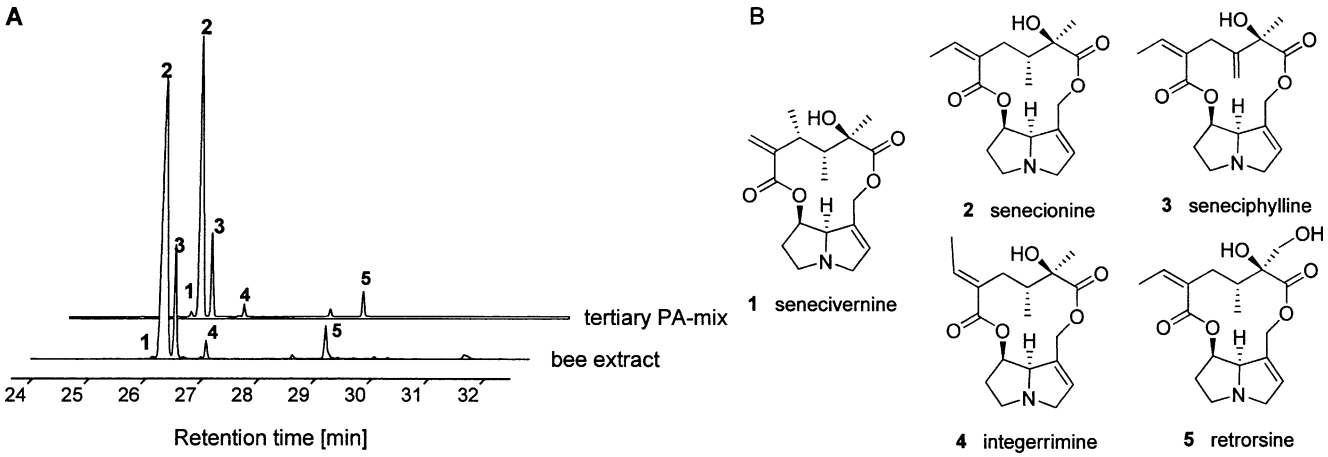

Fig. 6 Comparison of GC-NPD chromatograms (*A*) of the components (*B*) of the PA-mix (*A*, upper chromatogram) and a PA profile found in bees after feeding 2% PA-mix diet and 48 h test duration (*A*, lower chromatogram)

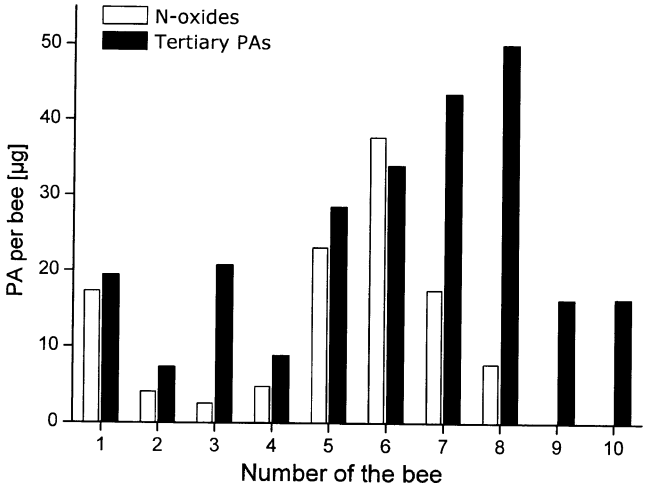

Fig. 7 Total amounts of PA-Nox and tertiary PA found in individual surviving bees after feeding 2% PA-Nox-mix

for consumers (Edgar et al. 2002; Kempf et al. 2008). The food supply of honey bees depends to a great extent on the acquisition of nectar and pollen from flowers. It is still an open question whether floral nectar of PA-plants naturally contains PAs, since PA contamination found in honey also could be caused by pollen. Any honey that was found to contain PAs was also found to be contaminated with pollen of PA-plants (Kempf et al. 2008). The genuine occurrence of PAs in floral nectars has been suggested frequently but never proved unequivocally (Detzel and Wink 1993; Gaffal and Gammal 2003; Singaravelan et al. 2005; Gegear et al. 2007). It seems that the alkaloid content of nectar generally is lower than in pollen (Detzel and Wink 1993). Since up to 3% of flowering plants contain PAs (Culvenor 1980), there is a high probability that honey bees are exposed to these potentially toxic compounds. So far, no data have been available to explain how bees accomplish this challenge.

Toxicity and Deterrence The first approach was to expose bees to a naturally occurring PA mixture. The PA-mix obtained from *S. vernalis* contained PAs of the senecionine-type, representing one of the most prominent and toxic classes of macrocyclic PAs (Hartmann and Witte 1995; Fu et al. 2004). The results of the feeding experiments with the 2% PA-mix showed high mortalities compared to the PA-free control. Monocrotaline showed a similar toxicity profile (increased mortality at >30 h).

Thus far, only the influence of heliotrine, a representative of PA monoesters of the lycopsamine type, has been tested on honey bees, and a LD_{50} of 0.1% was determined (Detzel and Wink 1993). Since the test conditions were quite different (this study: 2 h PA feeding pulse; Detzel and Wink: continuous feeding of a PA diet for 48 h), it seems understandable why we did not observe such high mortality rates in our experiments at PA concentrations of 0.2%. In fact, both experiments demonstrated that adult worker bees could safely handle quite high PA amounts in their diets. During the feeding period of 48 h, the bees did not defecate, and thus were unable to reduce the ingested PA load by excretion.

The observed PA concentrations among one distinct group of bees varied by almost one order of magnitude (Fig. 5, *B*) and demonstrated impressively the dimension of biological variation even under idealized laboratory conditions and among closely related individuals.

Strikingly, in the feeding experiment with the 2% PA diet, the average PA load of the surviving bees was markedly lower (50 µg/bee) as compared to the two groups of deceased bees (175 and 250 µg/bee, respectively; Fig. 5). The real PA load might be even higher, since our quantification measure only covered soluble and extractable PAs but not PAs bound to cellular structures or polar degradation or detoxification products. As a consequence, the results of the 2%-test might be interpreted as "the dose makes the poison", and bees that only cautiously sipped the highly contaminated diet were able to survive. Since we did not observe any noticeable effects in repellency or mortality for the experiment with the 0.2% PA diet, it seems that bees can deal safely with PA concentrations found in their environment. A similar result was found for the alkaloids nicotine/anabasine and caffeine, which did not deter bees when administered in natural concentrations. Nicotine and caffeine were even found to stimulate feeding (Singaravelan et al. 2005).

The design of the experiments allowed us to distinguish between mortalities caused by potential stress through starvation, possibly caused by deterrent effects of the food (Fig. 3), and mortality caused by the ingestion of pro-toxic tertiary 1,2- unsaturated PA (Figs. 2 and 4). The results indicate that the toxicity observed at high concentrations (2% PA diet) of the tertiary PA-mix or monocrotaline must be caused by the ingested PAs. PA-Noxs as well as tertiary PAs that lack the 1,2-double bond (i.e., 1,2-dihydromonocrotaline) were non-toxic. We, therefore, suggest that the toxic effects observed with 1,2-unsaturated tertiary PAs are caused by cytochrome P450-mediated bioactivation (see Introduction). The PA-Nox-mix showed a significant deterrence effect at a concentration of 2%. As mentioned, most potential nectar plants of bees (species of the Asteraceae and Boraginaceae) contain PAs mainly as *N*-oxides (Hartmann and Witte 1995; Boppré et al. 2005, 2008). The repellent effect may represent an essential adaptation of bees to recognize plant PAs and consequently to provoke individuals to prevent the ingestion of hazardous amounts.

Metabolism The conversion of PA-Noxs to tertiary PAs is usually regarded as an unspecific reduction (see Introduction).

Therefore, we analyzed how bees metabolize PA-Noxs. In all instances, the PA-Noxs were at least partially converted to the corresponding tertiary PAs in the intestines. The extent of conversion varied strongly, but the overall dose of toxic tertiary PAs never exceeded 50 μg tertiary PA/bee (Fig. 7). This was in accordance with the average content observed for the surviving bees in the feeding experiment with 2% PA diet (Fig. 5, *C*).

We also examined the ability of bees to convert pro-toxic tertiary PAs into non-toxic PA-Noxs. This reaction is a well-known detoxification mechanism that has been demonstrated for several specialized insects that sequester PAs from their food plants and utilize them for their own benefit (Hartmann and Ober 2008). We never detected any significant *N*-oxidation of tertiary PAs in bees (data not shown). Thus, detoxification of PAs by *N*-oxidation can be excluded as a mechanism of detoxification in bees. This is corroborated by the fatalities observed with bees at high 1,2-unsaturated tertiary PA concentrations (Figs. 2 and 4).

Trophallaxis Experiment Our experiments of the horizontal PA transfer among bees revealed that bees that ingested high amounts of PAs (feeding on 2% PA diet) donated only 4% of their load to other bees, while bees that ingested lower amounts of PAs (0.2% PA diet) transmitted more than 15%. Obviously, the receiving bees are able to recognize a possibly hazardous load brought in by the foraging workers. Speaking for the colony, this deterrent effect would be beneficial, since foraging bees with high PA loads are less likely to be unloaded in the hive and, therefore, are less likely to recruit new workers for their potentially dangerous nectar source (Gould and Gould 1995; Seeley 1995). Possibly, the same sensory system that elicits feeding deterrence in the field is also involved in the control of trophallaxis.

In summary, our results suggest that adult worker bees are not deterred by PA concentrations that reflect natural conditions. Beyond that, bees apparently tolerate natural PA contamination of their food without adverse effects. Important questions and consequences arise, however, when we consider the observed PA tolerance of individuals in respect to PA transmission by trophallaxis within the bee community. If bees do not discriminate PA-containing food *per se*, PAs would be a natural component of honey and could only be avoided by limiting the access of bees to PA-plants. Are bees able to protect their offspring from contact with PAs? The well-documented mutagenic effects of PAs would be even more harmful during early developmental stages of the larvae. Are there any chronic effects of low PA concentrations on the fitness of the colony? Further studies are in progress to address these questions. We expect that the answers will provide useful information as to what extent bees are adapted to handle naturally occurring toxins such as PAs and thus prevent detrimental effects on their colonies. Perhaps this will improve our understanding of the phenomenon of unexplained mortality of bees, sometimes observed by beekeepers.

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have shown that extracts of *L. bostrychophila* affect the settling behavior of conspecifics, with the deterrent effect depending on the concentration of the extract and the polarity of compounds in the extract (Green 2005). Recent work also has indicated that extracts of fungal colonies can affect the substrate chosen by *L. bostrychophila* (Green 2008), although the compounds responsible for this effect were not analyzed. So, *L. bostrychophila* can respond to chemicals in their environment. Before looking in more detail at the behavioral effect of compounds in their habitat, it is necessary to have a clearer idea of the role played by compounds produced by *L. bostrychophila*. Howard and Lord (2003) have identified several different fatty acids—ranging from C_{16} to C_{20}—and some fatty acid amides in extracts of the cuticle of *L. bostrychophila*, and they suggested that cuticular compounds could have a role in maintaining populations of *L. bostrychophila*. Although the behavioral effects were not investigated, they did find that cuticular compounds, in particular a fatty acid amide, stearamide, could inhibit the adhesion of certain fungal conidia to the cuticle (Lord and Howard 2004).

Fatty acids have been reported widely as components that are easily extracted from either the cuticle of insects (Jarrold et al. 2007; Gołębiowski et al. 2007, 2008a, b) or from the whole insect (Buckner and Hagen 2003; Reinhard et al. 2003). Fatty acid methyl esters are much less widely reported from insects (Moshitzky et al. 2003; Herman et al. 2005), especially since derivatization of the fatty acids (to more volatile methyl esters) prior to GC-MS does not allow the two different compounds to be distinguished. Fatty acids commonly encountered in insects, foods, or substrates can affect behavior of insects, attenuating the feeding-stimulant effect of sugars for *Diabrotica virgifera virgifera* (Bernklau and Bjostad 2008) and decreasing the re-mating behavior of female *Bombus terrestris* (Baer et al. 2000).

The aim of this study was to build on the work of Green (2005) and relate the behavioral effect of insect extracts (repellence) to the compounds extracted from *L. bostrychophila*. The most repellent extract of *L. bostrychophila* was fractionated, based upon the solubility of compounds in methanol and water, re-tested (to identify the most repellent fraction), and analyzed by GC-MS to see if some of the main compounds, particularly fatty acid amides, fatty acids, and fatty acid methyl esters, could be detected in the un-derivatized extracts, and whether these chemicals could help to explain the effects of the extracts. Thin layer chromatography was used to check for the presence of other lipids in fractions and extracts. Aside from providing more information on the chemical ecology of *L. bostrychophila*, there is the possibility that compounds identified in repellent extracts could be used to protect vulnerable areas from *L. bostrychophila*.

Methods and Materials

Insects The culture of *L. bostrychophila* was kept in a growth chamber (Vindon Scientific, UK) (76% RH, 28±2°C) on a 1: 1: 1: 1 mixture, by mass, of wheatgerm (Community Foods, UK); dried skimmed milk powder ("Marvel", Premier International Foods, UK), active yeast ("Quick Yeast"; Doves Farm, UK), and organic flour (Shipton Mill, UK). Insects were collected from the main culture by placing filter paper (5 cm diam) onto the surface of the diet media and allowing insects to crawl onto the paper. Insects were decanted into a 2 l ceramic dish and chilled for 10 min (5±2°C) before they were sieved (250 μm mesh) to retain adults and late stage nymphs. Insects were frozen (−18°C) and weighed (2.52 g).

Preparation of Insect-Extracts Four different extracts were tested for their effects on the settling behavior of *L. bostrychophila*. Sequential extracts of whole freshly collected insects were prepared in hexane (hexane) and aqueous 80% methanol (80%MeOH). The 80%MeOH extract was then sub-divided into two fractions based upon the compounds in the dried extract that would dissolve in either methanol (MFr) or water (WFr). First, sequential extracts of fresh sieved insects were prepared in 50 ml aliquots of hexane (10 min) and 80% methanol (24 h) (as described in Green 2005) with 12 h between extractions to allow evaporation of hexane. Each extract was filtered through an IST Isolute ® SPE frit (20 μm pore size), evaporated under a stream of nitrogen, and the amounts extracted into hexane (7.2 mg) and 80%MeOH (212 mg) were calculated. The 80%MeOH extract was divided into two fractions as follows. Methanol was added to 100 mg of dried extract, which was then agitated to dissolve as much material as possible. The sample was left to settle, and the supernatant was removed (MFr1), taken to dryness, and weighed (83 mg). The remaining solid was dried under a stream of nitrogen and weighed (WFr1, 17 mg). The remainder of the dried 80%MeOH extract (112 mg) was dissolved in 80% methanol to 10,000 ppm, 1,000 ppm, 100 ppm, and 10 ppm for bioassay. After bioassay and GC-MS analysis of this first sample a further 11 samples of booklice (2±0.7 g) were collected periodically over 1 month and extracted sequentially in hexane (mean extract weight=3.4±1.24 mg) and 80%MeOH (mean extract weight=311±101 mg). There was insufficient material in the individual hexane extracts for bioassay and for GC-MS analyses, so the hexane extracts for samples one to 12 were combined (44.5 mg). The proportions of the 12 samples extracted into hexane and 80%MeOH were 0.2±0.04% and 16±1%, respectively. Each of the 11 additional 80%MeOH extracts was fractionated, as described above, to yield methanol fractions (MFr2 to MFr12) and water fractions (WFr2 to WFr12). Sequential

extracts of the foods used to rear *L. bostrychophila* in culture—wheatgerm, dried skimmed milk powder, active yeast, and organic flour—were prepared in the same way to produce hexane and 80%MeOH extracts and then MFr and WFr.

GC-MS Analysis The hexane extracts and the methanol fractions of both insects and diet components were analyzed by GC-MS at 5,000 ppm. A Perkin-Elmer AutoSystem XL GC coupled to a Perkin-Elmer TurboMass MS (quadrupole) was used for analyses of samples. A 30 m×0.25 mm i.d.× 0.25 μm DB-5MS column (J. & W. Scientific, USA) was used with an oven program of 40–300°C at 3°C/min then 300°C for 4 min. The carrier gas was helium at a flow rate of 1 ml/min, and 1 μl injections (split 1:10) at 220°C were made by an autosampler. These injections equated to the extract from either 61 insects, for the combined hexane extract, or 0.9±0.07 insects for the MFr samples (N=12). Detection was by MS, fitted with an EI source operated at 70 eV with a source temperature of 180°C, and mass spectra were recorded in the range m/z 38–600 with 0.5 s scan time and 0.2 s inter-scan delay. The software was Turbomass, version 5.4.0. Peaks were integrated and peak-areas calculated for all detected compounds in each extract. Compounds contributing >2% of the total detected peak areas in this sample were putatively identified by comparison with published spectra in the NIST-database (Ausloos et al. 1992). Retention indices were calculated against an *n*-alkane series (C_{16} to C_{44}; Supelco, USA) so that the carbon chain length could be estimated for unknown compounds. The EI-spectra of compounds in the hexane extract and methanol fractions closely matched those of hexadecanoic acid, methyl ester (methyl palmitate; 16:0); (*Z*)-9-hexadecenoic acid (palmiteoleic acid; 16:1); *n*-hexadecanoic acid (palmitic acid; 16:0); (*Z*,*Z*)-9,12-octadecadienoic acid, methyl ester (methyl lineolate; 18:2); (*Z*)-9-octadecenoic acid, methyl ester (methyl oleate; 18:1); (*Z*)-9-octadecenoic acid (oleic acid; 18:1); (*Z*,*Z*)-9,12-octadecadienoic acid (linoleic acid; 18:2); and octadecanoic acid (stearic acid; 18:0). These eight compounds, together with octadecanoic acid, methyl ester (methyl stearate; 18:0) were purchased from Sigma-Aldrich, UK, dissolved to 10 mM in chloroform, and analyzed under the GC-MS conditions described above. Stearamide (octadecanamide) (Acros Organics) was analyzed as a standard to observe if it was present in the extracts.

Thin Layer Chromatography Methanol fractions (10 mg ml^{-1}) and the single hexane extract (1 mg ml^{-1}) were prepared. Marker compounds, dissolved in 2 ml aliquots of solvent, were either a mix of fatty acids (2 mg, 2.3 mg, 7 mg, and 3.2 mg, respectively, of C_{16}, $C_{16:1}$, C_{18}, and $C_{18:1}$ in chloroform); $C_{18:1}$ (2 mg in chloroform) or cholesterol (4.5 mg in acetone) (Sigma-Aldrich, UK). Aliquots (30 μl) were applied to an aluminum-backed analytical TLC-plate (20×20 cm, Kieselgel 60, Merck, Darmstadt, Germany) that had been activated by heating at 100°C for 1 h. The TLC plate was developed according to the method of Kupke and Zeugner (1978). First, the plate was run twice in chloroform (65); methanol (30); water (5), until the solvent front had risen 4 cm from the bottom of the plate (2.5 cm from the origin). The plate was dried, pre-saturated in solvent vapor and then developed in hexane (80); diethyl-ether (20); acetic acid (1.5) until the solvent front had migrated to 9.3 cm. For visualization, the plate was sprayed with 5% sulfuric acid in ethanol and charred at 200°C (Buckner and Hagen 2003). The Rf values for each spot were calculated. All extracts and samples were taken to dryness after use, and 5 ml of hexane were added before the vials were flushed with nitrogen to exclude air. Samples were then stored at −18°C.

Effects of Extracts, and Fractions Thereof, on the Settling Behavior of L. bostrychophila Several different experiments were conducted to observe the effects of extracts and compounds on the distribution-behavior of *L. bostrychophila*. The procedure was the same in each case. For the first set of experiments, using an 80%MeOH extract of whole *L. bostrychophila*, pairs of glass-fiber discs (Whatman GF/A grade, 2.1 cm diam) were laid onto a tray covered in aluminum foil, and aliquots (100 μl) of an 80%MeOH extract of booklice (10,000 ppm) were applied to one of the discs (treated), with 100 μl of 80%MeOH applied to the other (solvent blank). This was repeated for 1,000 ppm, 100 ppm, and 10 ppm concentrations. For the controls, solvent was applied to both discs, and for the purposes of comparison one was randomly assigned as treated and one as blank. Given that the weight of an individual insect—collected as described above—is 45±1.4 μg (Green 2005), it was possible to estimate the numbers of insects represented by 100 μl aliquots of the different concentrations of this 80% MeOH extract with ±3.1% margin of error. Once dry, each pair of discs was fixed 1.5 cm apart at the center of individual 9 cm Petri-dishes with solvent-free glue (Pritt Stick, Henkel Consumer Adhesives, UK), thus ensuring that insects could not get underneath the discs during the experiment. Dishes were made escape-proof with a 0.5 cm band of Fluon ® around the inside-rim. The Petri-dishes were equilibrated, at 28±1°C and 76±1.5% RH, in a growth chamber (Binder KBWF 240). Bioassays were conducted as described in Green (2008), except that saturated salt (NaCl) solution and plastic bags were not required to maintain humidity. The position of insects was recorded, after 12 h, by photographing the dishes with a digital camera (Sony® Cybershot™). Insects upon either of the two discs were designated as having selected, while insects remaining in the

dish had not selected. All dishes were placed in a freezer (−18°C) for 2 h, and the insects in each dish were counted: from this, the number of insects that were not on either disc (non-selecting) could be calculated. The MFr1 and WFr1 samples—approximated to their concentration in the 80% MeOH extract, 8,000 ppm and 2,000 ppm, respectively—also were presented to groups of *L. bostrychophila* separately and in combination to establish a link between the compounds detected in the GC-MS-analysis and behavior. A summary of the choices presented to groups of *L. bostrychophila* in the first set of experiments is shown in Table 1. The 11 additional 80%MeOH extracts were dried, re-dissolved in methanol, and the supernatant was tested at 8,000 ppm (MFr2 to MFr12).

Effects of Fatty Acids and Fatty Acid Methyl Esters on the Settling Behavior of L. bostrychophila The eight compounds identified in the extracts by GC-MS, plus methyl stearate, were dissolved in chloroform and 100 µl aliquots of 10 mM, 1 mM, 0.1 mM, 0.01 mM, and 0.001 mM were presented to groups of *L. bostrychophila* on glass fiber discs in treated vs. solvent-blank bioassays, using the method described above.

Statistical Analysis The *Kruskal-Wallis test* (SPSS, v.16.0.0) was used to compare the percentage of insects selecting among groups presented with different choices. *The Mann-Whitney U test* (SPSS, v.16.0.0) was used to compare the percentage of insects selecting between the control group and each experimental group. The percentage of selecting insects was sub-divided into those on the blank disc and those on the treated disc, and the proportions of insects selecting each disc—within each experiment—were compared with the *Wilcoxon signed ranks test*. All tests were two-tailed. All bioassay and chemical data are presented ± the standard error of the mean (SE), where appropriate.

Results

GC-MS Analysis Methyl palmitate, palmiteoleic acid, palmitic acid, methyl lineolate, methyl oleate, lineoleic acid, oleic acid, and stearic acid could be identified in most of the 12 methanol fractions (Fig. 1). The exception was sample MFr2, where the methyl esters of palmitic, linoleic, and oleic acids were not detected. Stearamide was not detected in any of the samples. The molar concentration of identified compounds ranged from 0.02±0.004 (methyl palmitate) to 1.2±0.11 mM (linoleic acid), equating to a mass of 7±0.45 ng and 260±32.4 ng per insect, respectively (Table 2). Fatty acids and fatty acid methyl esters made up approximately 30% and 95±2.4% (N=12) of the detected peaks in the hexane extract and methanol fractions, respectively. Furthermore, the proportion of the samples that were fatty acids and fatty acid methyl esters was lower in the hexane samples (3.36%) than in the methanol fractions (11.2±1.20%; N=12). All eight compounds were present in the combined hexane extract (Fig. 1c), but they were less concentrated and less material was extracted per insect when compared with the methanol fractions (Table 2). The NIST database gave equivocal results for the structures of the remaining compounds in the hexane extract, although a comparison of the retention times of compounds in the hexane extract with a standard alkane mixture indicated that the compounds eluting after 70 min were between 27 and 31 carbons in length (Table 3). Palmitic acid (0.33 µM), linoleic acid (1.7 µM), and oleic acid (0.44 µM) were detected in the 5,000 ppm hexane extract of wholewheat organic flour.

Table 1 Choices presented to *Liposcelis bostrychophila* in the first set of bioassays

Groups of insects were presented with 80% methanol extracts (80%MeOH) water fractions of the 80%MeOH extract (WFr1) and methanol fractions of the 80%MeOH extract (MFr1) at the concentrations indicated. Ten replicates of each treatment; 20 replicates of the control (solvent blank vs. solvent blank)

Extract on disc 1 and its concentration, ppm	Extract on disc 2 and its concentration, ppm
solvent blank	solvent blank
80%MeOH, 10,000	solvent blank
80%MeOH, 1,000	solvent blank
80%MeOH, 100	solvent blank
80%MeOH, 10	solvent blank
80%MeOH, 10,000	80%MeOH, 10,000
80%MeOH, 10,000	80%MeOH, 1,000
80%MeOH, 10,000	80%MeOH, 100
80%MeOH, 10,000	80%MeOH, 10
solvent blank	solvent blank
WFr1 2,000	solvent blank
MFr1 8,000	solvent blank
hexane, 10,000	solvent blank
MFr1 8,000	WFr1 2,000

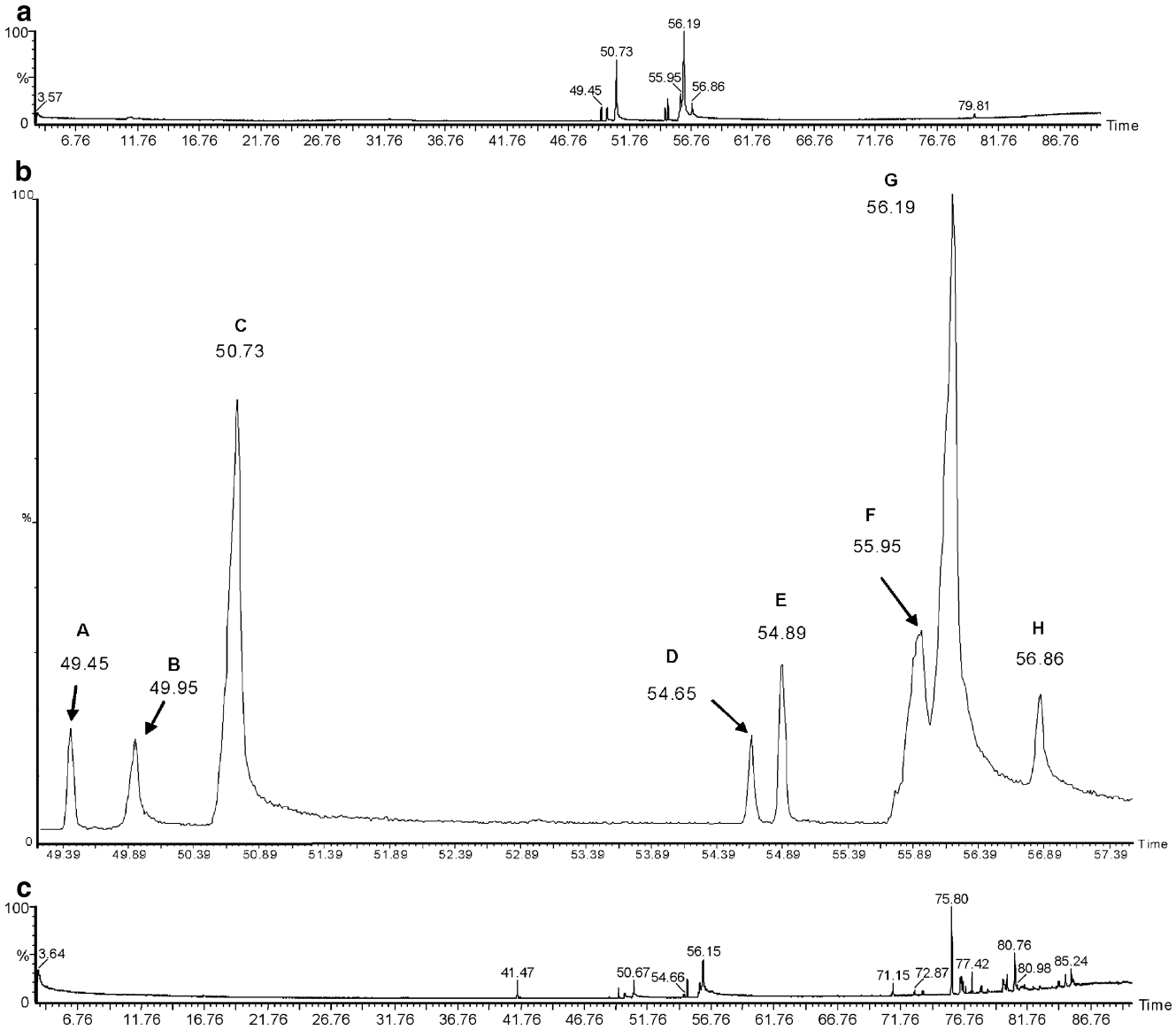

Fig. 1 GC chromatogram of a typical methanol fraction (MFr4) prepared from an 80% methanol extract of whole *Liposcelis Bostrychophila* (**a**); an expanded view of the region where the fatty acids and fatty acid methyl esters elute (**b**) and a GC-chromatogram from a hexane extract of whole *L. bostrychophila* (**c**) (**b**). *A* = methyl palmitate; *B* = palmiteoleic acid; *C* = palmitic acid; *D* = methyl lineolate; *E* = methyl oleate; *F* = linoleic acid; *G* = oleic acid and *H* = stearic acid

These molarities were equivalent to 0.68, 0.69, and 0.21% of their concentration in the 5,000 ppm hexane extracts of *L. bostrychophila*. Similarly, the hexane and methanol fraction of dried active yeast contained palmitic acid (0.33 μM): 0.11% and 0.68%, respectively, of the concentration in the 5,000 ppm insect samples. Fatty acids or their methyl esters were not detected in the hexane extract or methanol fraction of skimmed milk powder or wheatgerm.

Thin Layer Chromatography The methanol fractions and hexane extract contained fatty acids (Rf=0.48) and cholesterol (Rf=0.37). By comparing the relative positions of the marker-compounds with those in the fractions and extract and by reference to published data (Kupke and Zeugner 1978) it was possible to tentatively identify triacylglycerols in the MFr samples (Rf=0.80) and four putative phospholipids (Rf=0.08, 0.13, 0.18, and 0.26). Triacylglycerols and phospholipids were not detected in the hexane extract, although cholesterol esters (Rf=0.97) and two unknown compounds (Rf=0.99 and 0.62) were visible.

Effects of Extracts, and Fractions Thereof, on the Settling Behavior of L. bostrychophila Each 100 μl aliquot of the 80%MeOH extract (10,000 ppm) was equivalent to a density of 77 insects per cm^2 or 267 insects per disc, with the numbers decreasing proportionately with the concentration

Table 2 Compounds detected by GC-MS in either a hexane extract or in methanol fractions of *Liposcelis bostrychophila*

Compound	Hexane extract at 10,000ppm[a]			Methanol fractions at 8,000ppm[a]		
	Molarity, mM	Mass of compound, per insect, ng	Proportion of hexane extract, %	Molarity, mM (SE)	Mass of compound, per insect, ng (SE)	Proportions of methanol fractions, % (SE)
Methyl palmitate	0.01	0.002	0.02	0.02 (0.004)	2.7 (0.45)	0.06 (0.013)
Palmiteoleic acid	0.17	0.035	0.43	0.38 (0.06)	68.7 (11.84)	1.20 (0.184)
Palmitic acid	0.98	0.02	0.25	0.50 (0.06)	95.4 (15.6)	1.61 (0.203)
Methyl lineolate	0.004	0.001	0.01	0.02 (0.01)	3.8 (0.77)	0.09 (0.028)
Methyl oleate	0.028	0.007	0.08	0.05 (0.01)	9.7 (1.6)	0.20 (0.045)
Linoleic acid	0.482	1.1	1.35	1.2 (0.11)	260 (32.4)	4.50 (0.403)
Oleic acid	0.422	0.9	1.19	0.9 (0.11)	194.7 (34.33)	3.29 (0.416)
Stearic acid	0.006	0.002	0.02	0.07 (0.01)	13.7 (2.61)	0.24 (0.039)
			Total=3.36			Total=11.16 (1.202) [b]

[a] 5,000 ppm samples were analyzed by GC-MS, so the molar concentration of each compound has been scaled-up by a factor of 2 and 1.6 for the hexane extract and methanol fractions, respectively. The mass of compound per insect and proportions are independent of the extract concentration. The mean molarity; mass of compound per insect, and the proportions of fatty acids (FA) or fatty acid methyl esters (FAME) in the methanol fractions were calculated from 12 samples, except for methyl palmitate, methyl lineolate and methyl oleate (N=11)

[b] The methanol-soluble FA and FAME are 8.9±0.96% of the 80%MeOH extract (10,000 ppm), by mass

of extract. The effect of the insect extracts on settling behavior of *L. bostrychophila* depended on the choices presented. An 80%MeOH extract of whole insects (10,000 ppm) deterred settling if it was paired with a blank (untreated) disc (*Mann-Whitney U* test, $P<0.01$; Fig. 2). There was a point between 10,000 ppm and 1,000 ppm when this deterrent effect was lost (Fig. 2). The results were different when both discs were impregnated with extract. Groups of *L. bostrychophila* distributed equally between two discs that both had 10,000 ppm 80%MeOH extract applied (*Wilcoxon matched pairs test*, $P>0.05$; Fig. 2). Once there was an order of magnitude difference between the extracts applied to each disc (i.e., 10,000 ppm vs. 1000 or 10,000 ppm vs. 100) more of the insects settled on the disc to which the lower concentration was applied (*Wilcoxon signed ranks test*, $P<0.05$; Fig. 2), although the insects did not select between 10,000 ppm and 10 ppm (*Wilcoxon signed ranks test*, $P>0.05$). The choices presented also affected the overall level of selection, i.e., the proportions of insects selecting either disc, remaining constant with two treated discs. In contrast, when there was a choice between an untreated disc and treated disc, overall selection declined as the concentration of extract on the treated disc increased (*Mann-Whitney U test*, $P<0.01$; Fig. 3).

A 10,000 ppm hexane extract (equivalent to 3,530 insects per cm^2 or 12,230 per disc) deterred settling (*Wilcoxon signed ranks test*, $P<0.05$; Fig. 4) but less than a methanol fraction (MFr1) (*Mann-Whitney U test*, $P<0.01$; Fig. 4). Overall selection was not different between the hexane extract and MFr1 sample (*Mann-Whitney U test*, $P>0.05$). For comparative purposes, to reflect the levels of hexane- and 80% methanol-soluble compounds extracted from the insects, an 82-fold dilution of the hexane extract (120 ppm) was tested and found to affect neither the level of selection (*Mann-Whitney U test*, $p>0.05$) nor the choice of disc (*Wilcoxon signed ranks test*, $P>0.05$) (data not shown). The MFr1 (8,000 ppm) and WFr1 (2,000 ppm) samples deterred settling of *L. bostrychophila* (*Wilcoxon signed ranks test*, $P<0.05$; Fig. 4). The WFr1 sample was less deterrent than the MFr1 (*Mann-Whitney U test*, $P<0.05$; Fig. 4), which was confirmed when both MFr1 and WFr1 samples were presented to insects in the same dish (*Wilcoxon signed rank*, $P<0.01$; Fig. 4). There was variability in the proportions of insects selecting either disc among the MFr2 to MFr12 samples (8,000 ppm) (*Kruskal Wallis test*, 11 df, $P<0.01$), but the overall levels of selection for each sample were not different from a control

Table 3 Kovats indices and percentage peak areas associated with unknown compounds detected in a hexane extract of *Liposcelis Bostrychophila*

KI[a]	% peak area
2696	2.0
2840	16.2
2862	3.3
2864	2.8
2867	2.2
2891	3.7
2929	3.3
2967	4.7
2976	2.8
2994	8.5
3120	2.5
3134	3.2

[a] *KI* Kovats index. KI's were calculated by comparing the retention times of a standard alkane series with those of compounds in the hexane extract

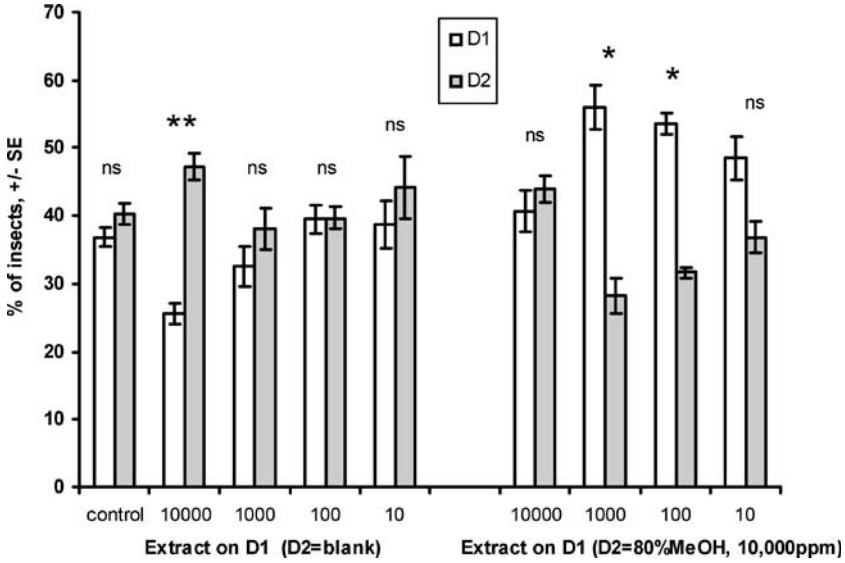

Fig. 2 Proportions of insects (%±se) selecting each disc when *Liposcelis Bostrychophila* were given a choice between either a treated disc (impregnated with different concentrations of an 80%MeOH extract) and blank disc or two treated discs that both had an 80%MeOH extract applied. The concentration of the 80%MeOH extracts of *L. bostrychophila* applied to D1 are in ppm. A comparison was made between the proportion of insects selecting each disc with the *Wilcoxon matched pairs test*, $^{**}P<0.01$; $^{*}P<0.05$ and *ns* = no significant difference. Each *bar* is the mean of ten replicates, except the control group ($N=20$)

group (*Mann-Whitney U test*, $P>0.05$). Each methanol fraction, including MFr2—in which methyl palmitate, methyl lineolate, and methyl oleate were not detected—deterred settling of *L. bostrychophila* in relation to a blank disc (*Wilcoxon signed rank test*, $P<0.01$) (data not shown). The level of deterrence was similar among the samples even though the number of insects represented by the methanol fractions varied between 112 and 188 (±3.1%) per disc.

Effects of Fatty Acids and Fatty Acid Methyl Esters on the Settling Behavior of L. bostrychophila Seven of the nine compounds tested repelled insects at 10 mM (*Wilcoxon signed ranks test*, $P<0.001$; Table 4), and repellence was associated with overall selection that was either increased (methyl palmitate, palmitic acid, methyl lineolate, and oleic acid) (*Mann-Whitney U test*, $P<0.001$ to <0.05; Table 5) or not different from the control (palmiteoleic acid, methyl oleate and lineoleic acid) (*Mann-Whitney U test*, $P>0.05$; Table 5). So, fatty acids and fatty acid methyl esters can affect the settling behavior of *L. bostrychophila*. The effect of each compound at concentrations <10 mM was different. The repellent effect was lost at 1 mM for all except methyl palmitate (*Wilcoxon signed ranks test*, $P<0.05$; Table 4), and the proportions of selecting insects either remained similar or increased. With the exception of linoleic acid, the fatty acids and fatty acids methyl esters were at concentrations below 1 mM in the repellent methanol fractions (Table 2). Methyl stearate—not detected in the extracts of booklice—was the only compound to deter settling of insects below 1 mM (*Wilcoxon Signed ranks test*, $P>0.05$; Table 4), although 0.1 mM palmiteoleic acid attracted *L. bostrychophila* (*Wilcoxon Signed ranks test*, $P<0.05$) and resulted in increased overall selection (*Mann-Whitney U test*, $P<0.05$; Table 5). Methyl lineolate and methyl oleate were the two other compounds that affected settling behavior of *L. bostrychophila*, with reduced overall selection when these two compounds were each presented at 0.01 mM (*Mann-Whitney U test*, $P<0.05$; Table 5).

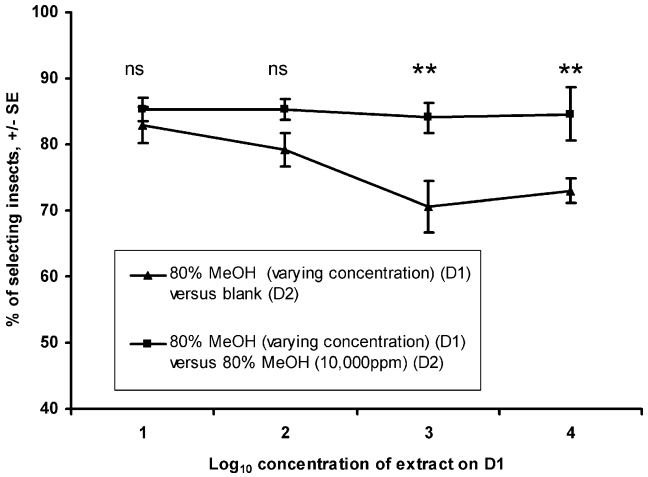

Fig. 3 Proportions of insects (%±se) selecting either disc when *Liposcelis Bostrychophila* were given a choice between either a treated and blank disc or two treated disc. 80% MeOH=80% methanol extract of *L. bostrychophila*, prepared after extraction with hexane. D1 and D2 are disc 1 and disc 2, respectively. Selection was compared between the proportions selecting using the *Mann Whitney U-test* $^{**}P<0.01$ and *ns* = no significant difference. Each point is the mean of ten replicates

Discussion

Extracts of *L. bostrychophila* deter settling of conspecifics, with the magnitude of the effect dependent on both the concentration of the extract and the solvents used to extract the material. Extracts of insects are known to affect the behavior of the same species, with the mixture of

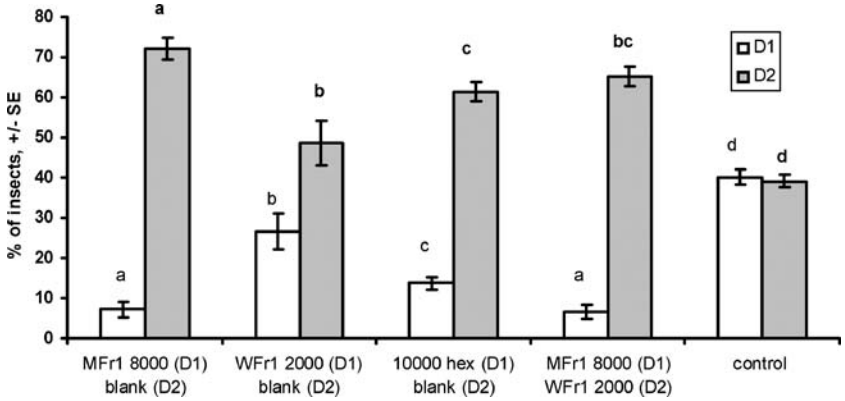

Fig. 4 Proportions of insects (%±se) selecting each disc when *Liposcelis Bostrychophila* were given a choice between either a treated disc (impregnated with MFr, WFr or a hexane extract) and blank disc or two treated discs to which either MFr or WFr had been applied. MFr1 8000 = methanol soluble fraction of an 80%MeOH extract at 8,000 ppm; WFr1 2000 = water-soluble fraction of an 80% MeOH extract at 2,000 ppm and 10,000 hex=10,000 ppm hexane extract. There was a difference among the proportions of insects selecting disc 1 (D1) and among those selecting disc 2 (D2) (*Kruskal-Wallis test*, $P<0.05$). Different *letters* indicate significant differences between the proportion of insects selecting disc 1 (letters in *normal text*) and disc 2 (letters in *bold*) (*Mann Whitney U-test*; $P<0.05$). The proportions of insects selecting either disc 1 or disc 2 within a treatment were different for all groups ($P<0.05$) except for the control (*Wilcoxon signed ranks test*). $N=10$ for all groups except the control ($N=20$)

compounds being particularly important in social insects for nest-mate recognition (Lucas et al. 2005) even though individual compounds in the extracts can act either as attractants or deterrents (Reinhard et al. 2003). *Liposcelis bostrychophila* is not a social insect but can occur in large groups that persist as long as resources (i.e., food and space) and a suitable microclimate permit. This persistence of groups may be modulated by physical and chemical

Table 4 Proportions of insects (%±se) selecting each disc when *Liposcelis Bostrychophila* were exposed to a disc treated with different concentrations of fatty acids or fatty acid methyl esters and a blank disc

	Concentration of compound on treated disc, mM				
Compound applied to each disc. none = solvent blank.	10	1	0.1	0.01	0.001
Methyl palmitate	6.3 (2.55)	25.8 (3.89)	34.6 (4.54)	36.3 (3.09)	nt
None	77.3 (2.55)***	56.3 (4.07)*	35 (4.49)	41.2 (3.95)	
Palmiteoleic acid	9 (1.44)	35.5 (3.43)	50 (2.68)*	36.1 (2.03)*	33.5 (3.34)
None	69 (3.06)***	41.4 (4.32)	31 (3.15)	20.2 (1.78)	43.2 (5.09)
Palmitic acid	47.3 (3.09)	42.4 (5.15)	38.3 (4.24)	33.8 (1.98)	39.1 (2.97)
None	42.8 (3.95)	43.5 (5.09)	34.1 (3.93)	35.9 (2.53)	44.6 (3.04)
Methyl lineolate	0.6 (0.33)	47.7 (4.41)	44 (3.87)*	29.1 (1.74)	36.5 (2.68)
None	89.6 (1.13)***	39.4 (4.29)	25.5 (4.29)	25.7 (2.53)	42.7 (3.05)
Methyl oleate	3.5 (0.60)	33.5 (2.65)	40.7 (3.13)*	28.4 (3.47)	34.3 (2.17)
None	69 (2.22)***	41.4 (3.57)	31.0 (2.61)	20.2 (1.43)	43.2 (2.64)
Linoleic acid	3.8 (0.64)	33.3 (4.39)	37 (2.38)	35.8 (2.76)	nt
None	78.4 (3.47)***	47.8 (4.82)	32.6 (2.85)	42.4 (3.61)	
Oleic acid	23.6 (3.49)	39.4 (4.35)	33.7 (3.66)	37.7 (2.65)	nt
None	66.5 (4.32)***	47.2 (5.71)	38.2 (4.65)	40.2 (2.42)	
Stearic acid	35 (6.06)	44.5 (2.48)	35.7 (4.06)	34.9 (3.15)	35.5 (2.04)
None	45.6 (5.47)	44.9 (2.30)	45 (3.99)	45 (2.72)	48.3 (2.09)
Methyl stearate	20.5 (2.52)	44.3 (3.45)	36.1 (4.25)	29.5 (1.35)	39.4 (2.93)
None	60.2 (2.86)***	44.9 (3.17)	36.6 (5.35)	37.7 (1.70)*	40.4 (2.64)

The proportions of insects selecting each disc. within a treatment, were compared with the *Wilcoxon matched pairs test*. [$N=10$ replicates, except 0.01 M palmiteoleic acid ($N=20$) and control ($N=100$)]. The proportions of insects on each of the control discs were 39.8±1.24% and 36.9±1.22%

nt not tested

***$P<0.001$; *$P<0.05$

Table 5 Proportions of insects (%±se) selecting either disc when *Liposcelis Bostrychophila* were exposed to choices between discs treated with different concentrations of fatty acids or fatty acid methyl esters and a blank disc

	Concentration of compound on treated disc, mM				
Compound on treated disc	10	1	0.1	0.01	0.001
None (control)			76.7 (1.21)		
Methyl palmitate	83.6 (2.61) *	82.1 (1.99)	69.6 (3.07) *	77.6 (1.62)	nt
Palmiteoleic acid	73.4 (2.64)	70.9 (4.45)	84.2 (1.72) *	56.7 (1.88) ***	79.0 (3.21)
Palmitic acid	89.3 (1.08) ***	85.9 (0.97) **	72.5 (2.37)	69.8 (3.47)	83.7 (2.18) *
Methyl lineolate	90.2 (1.26) ***	87.1 (1.17) **	69.5 (4.18)	54.8 (2.67) ***	79.2 (1.88)
Methyl oleate	72.5 (2.33)	74.9 (2.52)	71.7 (2.91)	48.6 (3.52) ***	77.5 (2.66)
Linoleic acid	81.5 (3.44)	82.6 (2.10)	69.6 (1.44) *	78.2 (3.31)	nt
Oleic acid	90.1 (1.21) ***	86.6 (3.26) **	71.8 (3.01)	78.2 (2.07)	nt
Stearic acid	80.6 (2.92)	89.4 (2.22) ***	80.7 (3.05)	79.9 (2.87)	83.8 (2.04) *
Methyl stearate	80.7 (2.76)	89.2 (1.71) ***	72.2 (3.64)	67.1 (2.02)**	79.8 (1.58)

The overall level of selection of either disc in the control group (N=100 replicates) was compared with that of each other treatment [N=10 replicates, except 0.01 M palmiteoleic acid (N=20)] using the *Wilcoxon Mann Whitney U-test*

nt not tested

***P<0.001; **P<0.01; *P<0.05

interactions among individuals. Chemicals from large groups of individuals might build up to repellent levels, causing dispersal. The settling behavior of *L. bostrychophila* appears to be governed by a balance between the repellence exerted by high concentrations of extract (i.e., large numbers of insects) and the proximity of an area with either fewer or no insects. This suggests that the choices could exert a push-pull effect (Miller and Cowles 1990; Cook et al. 2007). The fact that *L. bostrychophila* did not differentiate between two repellent extracts until there was at least an order of magnitude difference between the concentrations of extract on each disc provides additional support for this hypothesis. When there was a choice between two repellent extracts (i.e., 80%MeOH, 10,000 ppm), effectively two extracts with a push-effect, overall selection was not affected when compared with a control group. It is possible that the regulation of settling behavior by short-range chemical cues can be disrupted by the build-up of volatiles emitted from concentrated extracts, as suggested by Green (2008). The effect of volatile compounds on the behavior of *L. bostrychophila* requires clarification by further study.

Hexane extracted less material from samples of *L. bostrychophila* than 80%MeOH, so although the hexane extract repelled settling of *L. bostrychophila* at 10,000 ppm it represented considerably more insects per disc than either the 80%MeOH extract or the 8,000 ppm methanol fractions. Insect extracts usually are prepared in apolar solvents, such as hexane (Kaib et al. 2002; Roux et al. 2006; Scott et al. 2008) or chloroform (Nelson and Charlet 2003), and as a result, hydrocarbons (straight chain saturated/unsaturated and methyl branched) predominate in the extracts (Howard and Blomquist 2005; Juárez and Fernandez 2007). Similarly, as yet unidentified long chain (>C_{27}) hydrocarbons were the main compounds detected in the hexane extract of *L. bostrychophila*.

Within the deterrent methanol fractions, saturated, mono-enoic, and dienoic fatty acids and fatty acid methyl esters with between 16 and 18 carbons were found to dominate the compounds detected by GC-MS. Although fatty acids and fatty acid methyl esters were present in the hexane extract, they were at a much lower concentration. Palmitic, oleic, and linoleic acids are widely thought to be the major fatty acids present in insects (Stanley-Samuelson et al. 1988), and while it is less usual to find these compounds with fewer than 16 carbons atoms or more than 18, there are exceptions (Thiéry et al. 1995; Jarrold et al. 2007; Gołębiowski et al. 2008a). Fatty acids were extracted from wholewheat flour and yeast, and were detected in the hexane extract (palmitic, linoleic, and oleic acids) or the methanol fraction (palmitic acid). These three fatty acids could have been extracted from the undigested diet within booklice or from particles of diet sticking to the surface of individuals, although they have been identified previously in hexane extracts of the cuticle of booklice (Howard and Lord 2003). Other fatty acids and fatty acid methyl esters present in the extracts of whole booklice are either not extracted from the diet-components by hexane and 80% methanol, or are products of the metabolism of *L. bostrychophila*. It is not uncommon for there to be qualitative differences in the profiles of fatty acids between insects and their diet (Bashan et al. 2002; Chamberlain and Black 2005; Cakmak et al. 2007).

At the highest concentration tested (10 mM), the methyl-esters of palmitic- and stearic acids were repellent, as were all the other unsaturated fatty acids and their derivatives, whereas

the saturated fatty acids (palmitic and stearic) did not affect repellence or overall selection. Similarly, unsaturated fatty acids have been found repellent to ants (Howard et al. 1982; Dani et al. 1996), to boll weevils (Bird et al. 1987), and to deter the oviposition of mosquitoes (Hwang et al. 1984), while saturated fatty acids did not have any effect. Fatty acid methyl esters affect sexual signaling of *Tegenaria atrica* (Araneae) (Prouvost et al. 1999) and have a role in signaling in honey bee colonies (Trouiller et al. 1992). Other species of insect can be attracted or repelled by fatty acid methyl esters that are not necessarily of insect-origin (Singh et al. 1985; Bird et al. 1987; Henderson et al. 1991). The absence of fatty acid methyl esters from one of the methanol-soluble fractions did not reduce the repellence of the extract, indicating that these compounds are not essential for repellence, although the individual compounds repelled *L. bostrychophila* at 10 mM.

At 10 mM, the compounds were between 8- (linoleic acid) and 600-fold (methyl palmitate) more concentrated than found in the repellent methanol fractions. Most of the fatty acids and fatty acid amides did not affect the settling of *L. bostrychophila* at concentrations approaching those found in the insect extracts: strongly suggesting that a combination of compounds is likely to be responsible for the effects of extracts. Interaction among a mixture of linoleic, stearic, and oleic acids is a factor in host-recognition by larvae of *Diabrotica virgifera*, with a combination of these compounds as attractive as a crude fraction of maize seedlings (Hibbard et al. 1994). Fatty acids also can affect ovipositing insects by either stimulating or inhibiting egg-laying (Horikoshi et al. 1997; Parr et al. 1998; Li and Ishikawa 2006). Mixtures of fatty acids derived from eggs of *Cydia pomonella* (Thiéry et al. 1995) and *Lobesia botrana* (Gabel and Thiéry 1996) inhibit further egg-laying on a substrate. Behavioral effects are not exclusive to mixtures of fatty acids, as individual compounds, for example octanoic (caprylic) acid, determine orientation to—and oviposition on—host plants (Legal et al. 1999). The oviposition behavior of *L. bostrychophila* was not investigated, but it follows that if an organism is deterred from an area—whether there is any additive deterrence to oviposition or not—the number of eggs laid will be reduced.

For the purposes of protecting a vulnerable area and minimizing re-infestation, an ideal compound would be one that is highly repellent, causing maximal overall selection in the local area with most—or ideally all—insects choosing the untreated disc. All compounds at 10 mM, except palmitic and stearic acids, would fit into this category. As a treatment to include in a trap, it would be desirable to have increased overall selection with few or no insects on the blank disc. Palmiteoleic acid (0.1 mM) is the most significant compound with this effect, although methyl oleate (0.1 mM) is marginally attractive to *L. bostrychophila*. The use of these compounds as attractants or deterrents would require care to avoid contamination of the area to be protected, especially since monitoring and trapping would be carried out adjacent to foodstuffs or precious museum collections.

The extracts and compounds tested above were derived from *L. bostrychophila*, but there are chemicals in the habitat of booklice that are not of insect origin, especially those compounds that emanate from the stored products that are infested. Fungal hyphae produce palmitic, stearic, oleic, and linoleic acids, and the concentration of these compounds varies both over time and between species (Stahl and Klug 1996). *Liposcelis bostrychophila* respond to differences in fungal chemistry, selecting between extracts of different species (Green 2008), and it could be that the fatty acid composition of these extracts is responsible for this effect. The balance of repellent and attractant fatty acids in fungi might affect which species is selected as food by *L. bostrychophila*, as has been suggested for some species of mycophagous collembola (Nilsson and Bengtsson 2004). Therefore, extracts and fatty acids either repel *L. bostrychophila* as a result of the density of insects that they represent, or the insects move away because the insects do not recognize the mixture of compounds on the discs as a suitable food.

In summary, the results of this study could have a practical application, but they help also to explain more of the chemical ecology of *L. bostrychophila*, building on previous work (Howard and Lord 2003; Lord and Howard 2004; Green and Turner 2005; Green 2005, 2008). Compounds that were not detected by GC-MS constituted between 89 (methanol fractions) and 97% (hexane extract) of the samples. By using thin layer chromatography, it was possible to identify some of these unknown compounds, such as cholesterol, and to tentatively identify phospholipids and triacylglycerols, which are likely to have been extracted from inside the insect. It is not yet known how the different fatty acids and fatty acid methyl esters interact either with each other or with other compounds extracted from the insect. Further work will investigate the effect of combinations of fatty acids and fatty acid methyl esters on settling behavior of booklice; will seek to identify other compounds in the insect extracts; and will look for compounds left behind as *L. bostrychophila* move around their habitat to see whether these could have a function in governing settling and dispersal of populations.

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Holst, 1942) as an important natural mechanism of disease resistance in honeybee colonies. One of the most important aspects of hygienic behavior is that individual bees must detect and respond to appropriate stimuli from the diseased larvae early in the progression of the infection, before the pathogen becomes infectious. In this way, the quick and efficient detection and removal of diseased larvae by many bees prevents disease transmission throughout the colony.

Specifically, in this study, we investigated the hygienic response of honeybees to the fungal pathogen, *Ascosphaera apis*, which causes chalkbrood disease in larvae (Gilliam et al., 1983). Chalkbrood infection begins in the gut of a larva and grows slowly outward until it penetrates the cuticle. This typically occurs after a fifth instar has been capped, but before pupation begins. At this point, rapid growth and sporulation of the fungus occurs (Gilliam and Vandenberg, 1997). When adult bees remove infected larvae after the fungus has penetrated the cuticle, spores are ingested by adult bees and fed back to healthy larvae in the bee food. The healthy larvae ingest the spores, and the infection is perpetuated. Therefore, for hygienic behavior to be effective against chalkbrood, adult bees must remove the infected larva before the fungus has penetrated the cuticle.

Previous studies have suggested that the detection and subsequent removal of diseased larvae are guided by olfactory stimuli (Masterman et al., 2000, 2001; Gramacho and Spivak, 2003; Spivak et al., 2003). Differences in the degree of hygienic behavior at the colony level result from the relative speed and efficiency with which individual bees perform the behavior (Arathi et al., 2000, 2007; Arathi and Spivak, 2001). A response threshold model was postulated to describe the differences in colony-level expression of the behavior (Arathi and Spivak, 2001). In the laboratory, electrophysiological and behavioral conditioning of the proboscis-extension response (PER) were used to demonstrate that individual bees, from colonies selectively bred for hygienic behavior (see Methods), can detect larvae infected with chalkbrood, and discriminate between diseased and healthy brood at lower stimulus levels, compared to non-hygienic bees (Masterman et al., 2001; Spivak et al., 2003; for review, Fahrbach and Mesce, 2005). These studies, however, did not identify the specific volatile chemicals that bees use to detect diseased larvae, and did not test whether the application of such volatiles to healthy larvae could elicit an appropriate behavioral response in the field or not. In the present study we tested two hypotheses: 1) that hygienic behavior of honey bees can be elicited in field colonies by experimental application of appropriate olfactory stimuli; and 2) that hygienic behavior is based on a threshold response, in which colonies containing bees with the highest olfactory sensitivity to diseased brood initiate the hygienic response more quickly compared to colonies containing bees with a lower olfactory sensitivity.

Methods

Breeding The breeding program for colonies that display hygienic behavior began in 1993 at the University of Minnesota. Rapid-hygienic and slow-hygienic lines of bees (*sensu* Wilson-Rich et al., 2009) were selected from colonies derived from the Italian subspecies *Apis mellifera ligustica*. Colonies were selected initially based on their abilities to survive winter in Minnesota, to increase the population rapidly in spring, to produce large amounts of honey, and to be gentle (not sting) during normal management. If the colonies displayed the above set of traits, they were tested for hygienic behavior using a freeze-killed brood assay (Spivak and Reuter, 1998), which is an indirect measure of a colony's hygienic behavior. The removal of freeze-killed brood is correlated with the removal rates of diseased brood; colonies that uncap and remove >95% of freeze-killed pupae within 24 h will tend to remove diseased brood rapidly. Colonies that exhibited the most rapid removal rates were chosen to raise queens for the next generation. Daughter queens then were instrumentally inseminated with semen from drones of other rapid-hygienic colonies. The criterion for choosing rapid-hygienic colonies for breeding is an arbitrary cutoff established at 95% removal of freeze-killed brood within 24 h. Freeze-killed brood tests of many colonies yield a continuum of values, with colonies that remove a higher proportion of freeze-killed brood having higher degrees of hygienic behavior.

Volatile Collection Volatiles were collected from bees for two reasons. The first was to identify the changes between healthy and diseased brood. Three types of larvae were used: a) healthy fifth instars, b) fifth instars in the early stage of chalkbrood infection, and c) larvae fully overcome with fungal mycelia (mummies). Early stage chalkbrood-infected larvae were identified under a dissecting scope by a subtle discoloration of the larval cuticle, with no evidence of visible sporulation. Each type of larva was removed from the comb with sterile forceps and placed in quick-fit glass aeration chambers (46-cm-long × 19-cm-wide, RodaViss chambers, ARS, Gainesville, FL, USA). The disease was confirmed by fungal growth through the larva after the volatile collections were completed. Collections were determined to be from healthy larvae if no larvae showed clinical symptoms of disease at the conclusion of the volatile collection period. Each collection consisted of: a) 25 healthy fifth instars, b) 25 early-stage infection larvae, or c) 75 mummies. The second reason for collecting volatiles was for use in the gas chromatography-electroantennogram (GC-EAD) experiment. For this, volatiles were collected from a large quantity of healthy larvae or from diseased larvae placed in a larger aeration chamber (80-cm-long × 40-cm-wide, RodaViss chambers, ARS, Gainesville, FL, USA).

For each experiment, the volatile collection apparatus was constructed as follows: charcoal-filtered and humidified air at 0.5 Lmin^{-1} was passed over the larvae and then through pre-conditioned Super-Q adsorbent (30 mg, Grace, *nee* Alltech, Deerfield, IL, USA) traps for 24 h at room temperature. Each trap was eluted with 150 μl methylene chloride forced through the absorbent with filtered nitrogen gas. Elutions were stored at -70°C until used.

Chemical Analysis To identify the disease-related volatiles, chromatograms from the healthy, early-stage infected and fully diseased brood were compared. For the purposes of identification, 174 ng of butyl butyrate, as an internal standard, were added to 40 μl of extract, with 1μl samples of extract analyzed by gas chromatography-mass spectrometry (GC-MS) on an HP-6890 (Agilent Inc., Palo Alto, CA, USA) gas chromatograph linked to an HP 5973 mass spectrometer in the electron impact mode (70 eV, Agilent, Palo Alto). An HP-1 column (30 mm × 0.25 mm ID × 0.25 μm, J & W Scientific, Folsom, CA, USA), with helium as carrier gas, was used for the analyses; injection was splitless. The oven temperature was held at 50ºC for 5 min, then increased at 5ºC min^{-1} to 110°C, then 25°C min^{-1} to 230°C and held at this temperature for 10 min. Volatile compounds were confirmed by comparison of their chromatographic retention times and mass spectra with commercially available standards analyzed on the same instrument.

To isolate compounds perceived by bees, GC-EAD was used for the diseased-brood samples, the healthy larvae samples, and the standards. Aliquots (4 μl) of extracts were analyzed with an Agilent 6890 N gas chromatograph equipped with a TR-Wax column (30 m × 0.32 mm ID × 0.25 μm; Thermo Electron Corp, Belefonte PA, USA). This column permitted better separation of the compounds of interest than the HP1 column. Injection was splitless with helium as carrier gas. The oven temperature was held at 50°C for 5 min, then increased by 5°C min^{-1} to 110°C, then by 25°C min^{-1} to 200°C, and held at this temperature for 5 min. The effluent was split (SGE splitter, SGE Incorp., Austin, TX, USA) to allow simultaneous recording of the flame ionization detector (FID) and the EAD responses. Nitrogen was used as a make-up gas for the FID and as an auxiliary make-up gas (~15 mL min^{-1}) to carry the effluent to the EAD. GC-EAD Pro (Syntech, Hilversum, Netherlands) was used for simultaneous recording of the FID and EAD signals.

EAD recordings were obtained from excised antennae of bees that were 15–20 days old, as this is the age of bee that performs hygienic behavior (Arathi et al., 2000). In 2007, bees of known age were obtained by placing frames with brood ready to emerge in a cage in an incubator for 24 h. Emerged bees were marked with enamel paint on the thorax and returned to the source colonies for collection 15–20 days later. Three source colonies used for the GC-EAD tests were from the University of Minnesota breeding program and were deemed greater than 95% hygienic based on freeze-killed brood tests. The antennae were held between stainless steel electrodes (Syntech), and connections to the electrodes were made with conductivity gel (Spectra Gel® 360, Parker Laboratories, Fairfield, NJ, USA). A humidified air stream, containing the GC effluent, was delivered at 20 ml min^{-1} through a 44-cm glass tube to the antenna.

Field Bioassay I. Topical Applications The topical application assay tested the hypothesis that hygienic behavior of honey bees can be elicited in field colonies by experimental application of appropriate olfactory stimuli to healthy larvae. The assays were conducted in August 2007. Twelve colonies were chosen for the bioassay based on the freeze-kill removal method (one of the source colonies for the GC-EAD analyses also was used in this assay). A comb containing larvae was removed from each of the twelve colonies, and the position of 25 fifth instars on the comb was marked on a transparency overlaying the comb. A Picospritzer II (General Valve Corporation) was used to dispense 0.5 μl of each treatment onto healthy larvae. Five treatments were applied to separate groups of 25 healthy larvae: benzyl alcohol (Sigma-Aldrich, St Louis, MO, USA), 2-phenylethanol (Fluka, Steinheim, Germany), phenethyl acetate (Sigma-Aldrich, St. Louis, MO, USA), deionized water, and a mixture of equal parts benzyl alcohol, 2-phenylethanol, and phenethyl acetate. The compounds were drawn up by capillary action into a 5 μl capillary tube, and a 3 msec pulse of air at 80 psi was used to puff the compound onto a given larva. Neighboring larvae were not treated. The comb then was returned to the colony from which it was taken. At 4 h and 24 h, the transparency was replaced on the frame, and the number of larvae that the adult bees had removed was recorded.

Field Bioassay II. Paraffin Larval Dummies The assay utilizing paraffin larval dummies tested the hypothesis that hygienic behavior is based on a threshold response in which bees with the highest olfactory sensitivity to diseased brood initiate the hygienic response more quickly compared to bees with a lower olfactory sensitivity. Paraffin larval dummies were made by melting paraffin wax at 60–65°C with a 10^{-2} dilution of methyl linolenate (Acros Organics, NJ, USA). Methyl linolenate is one of several compounds present in brood pheromone of fifth instar larvae. When this compound is added to a paraffin wax dummy and placed in a larval cell, adult bees cap the cell containing the dummy (Le Conte et al., 1990). The addition of chalkbrood volatile compounds to wax dummies that contain brood pheromone

should result in a decrease in capping of these dummies, if adult bees detect and respond to the disease-related compounds. Bees are unable physically to remove paraffin dummies from the cell at nest temperatures (32–34°C), because the paraffin is soft and sticky. Therefore, in this assay, when brood pheromone was added to the paraffin dummy, and bees placed a wax capping over the dummy, it was an indication that the adult bees accepted the 5th instar as normal. When both brood pheromone and disease volatile(s) were added to the paraffin, and the bees did not cap the dummies with wax, it was assumed that bees detected the disease volatiles and exhibited hygienic behavior.

The response threshold model was tested by measuring the differential abilities of adult bees to detect chalkbrood compounds at differing concentrations. Using the mixture of wax and methyl linolenate, a total of eight treatments was formulated: phenethyl acetate at 10^{-2} (v/v) and 10^{-9}, 2-phenylethanol at 10^{-2} and 10^{-9}, benzyl alcohol at 10^{-2} and 10^{-9}, and a mixture of the previous three compounds at 10^{-2} and 10^{-9}. The concentrations were chosen based on differences in the frequency of capping observed in the preliminary development of this assay. Additional treatments consisted of methyl linolenate (in wax) alone and of paraffin wax without the addition of methyl linolenate or disease-associated compounds. The liquid wax containing each treatment was poured into plastic drinking straws (0.5 cm diam) and allowed to cool and harden. The straw then was cut away from the wax, and the wax was cut into 25 mm sections to create larval dummies.

The assays were conducted in August 2008. Six colonies that displayed varying degrees of hygienic behavior, ranging from 39% to 99%, as determined through freeze-killed brood assays (see Fig. 3), were chosen for this bioassay. A comb containing fifth instars was removed from the colony. Combs with fifth instars were chosen so that the larval dummies would be in context with actual fifth instars that were about to be capped with wax by adult bees. Twenty larval dummies of each treatment were placed into empty cells, and their location was marked on a transparency. No more than two treatments were tested on a single comb at a time. The combs were then returned to the colony and the number of dummies the bees capped with wax was recorded after 24 h.

Statistical Analysis Binomial regressions were performed to compare the proportion of healthy topically treated larvae removed or paraffin dummies left uncapped in response to each treatment, with the degree of hygienic behavior of the colony as a covariate. The delta method was used to calculate the student's t value for contrasts between treatments with differences declared at $\alpha=0.05$. Analyses were done with *Arc* (Cook and Weisberg, 1999).

Results

Chemical Analysis Mass chromatograms of volatiles collected from healthy fifth instars and larvae in early stages of chalkbrood infection are shown in Figure 1. Three peaks associated with diseased brood were non-detectable in collections from the healthy brood; these peaks appeared in the early stage diseased larvae and increased in quantity in those fully diseased. GC-MS analyses identified these compounds as phenethyl acetate, 2-phenylethanol, and benzyl alcohol. GC-EAD analyses of the volatiles of larvae in the early stages of chalkbrood infection showed that bee antennae responded consistently to these compounds (Fig. 2).

Field Bioassay I. Topical applications Colonies with higher degrees of hygienic behavior removed more treated larvae at 4 h ($t=4.844$, $df=54$, $P<0.001$) and at 24 h ($t=8.917$, $df=54$; $P<0.001$) compared to colonies that displayed slower hygienic behavior. There were no significant differences in the slopes of the regression lines among treatments; all colonies responded similarly to all treatments 4 h and 24 h after treatment (interaction between treatment and hygienic behavior for all; $P>0.05$).

At 4 h, most larvae treated with 2-phenylethanol, benzyl alcohol, the mixture, and water were not removed. However, more larvae treated with phenethyl acetate were removed compared to all other treatments: 2-phenylethanol ($t=4.485$, df=54, $P<0.001$), benzyl alcohol ($t=4.483$, df=54, $P<0.001$), the mixture ($t=6.181$, df=54, $P<0.001$), and water ($t=3.557$, df=54, $P<0.001$). Phenethyl acetate removal rates ranged from 0 to 84%.

At 24 h, there were significant differences in removal among all treatments (Table 1 and Fig. 3). Larvae treated with phenethyl acetate were removed in greatest numbers followed by larvae treated with the mixture, 2-phenylethanol, benzyl alcohol, and water (Fig. 3).

Field Bioassay II. Paraffin Larval Dummies None of the colonies capped paraffin dummies that lacked brood pheromone. The addition of the brood pheromone component, methyl linolenate, to the dummies resulted in a significant increase in capping. Capping of brood pheromone dummies ranged from 70% to 95%.

As predicted, when dummies were treated with both brood pheromone and one or a mixture of the disease volatiles, the bees in all colonies capped significantly fewer dummies treated with higher concentrations (10^{-2}) of the diseased larval compounds, as compared to dummies treated with lower concentrations (10^{-9}). This was true for all compounds: phenethyl acetate ($t=4.437$, $df=9$, $P<0.001$), 2-phenylethanol ($t=9.778$, $df=9$, $P<0.001$), benzyl alcohol ($t=7.388$, $df=9$, $P<0.001$), and the mixture

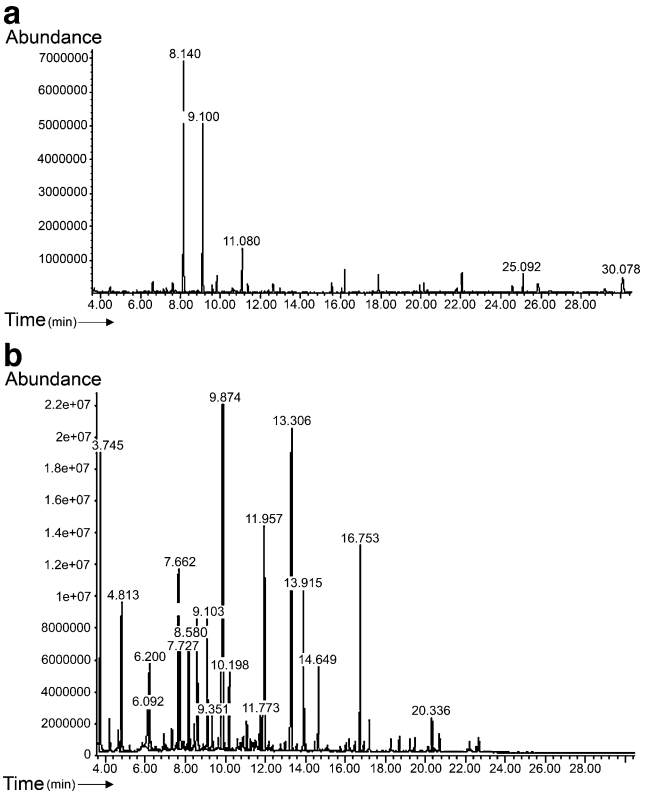

Fig. 1 Representative total ion mass chromatograms of volatile chemicals released by a) healthy 5th instar honeybee larvae and b) 5th instar larvae in early stages of chalkbrood infection. Retention time (min) of compounds of note: 8.14- butyl butyrate (IS), 8.58- benzyl alcohol, 9.1- (*E*)-β-ocimene, 9.874- 2-phenylethanol, 11.957- phenethyl acetate

(t=3.138, df=9, P=0.001) (Fig. 4). Colonies with higher degrees of hygienic behavior capped significantly fewer larval dummies (P<0.001). There were no significant differences in the slopes of the regression lines among treatments in the paraffin experiment, and the interaction between treatment and hygienic behavior, for all treatments, was not significant (P>0.05).

At the higher concentration (10^{-2}), there was no significant difference among the number of dummies capped for the treatments phenethyl acetate, the mixture, and paraffin alone. Dummies treated with phenethyl acetate, the mixture, and paraffin were capped significantly less than those treated with 2-phenylethanol and benzyl alcohol. All dummies treated with compounds at the 10^{-2} concentration were capped significantly less than those treated with the brood pheromone component alone, methyl linolenate (Table 2).

At the lower concentration (10^{-9}), dummies treated with phenethyl acetate and the mixture were capped significantly less frequently than dummies treated with methyl linolenate, 2-phenylethanol or benzyl alcohol. There was no difference in the numbers of dummies capped between the treatments methyl linolenate alone and 2-phenylethanol, methyl linolenate alone and benzyl alcohol, and between 2-phenylethanol and benzyl alcohol. There was no significant difference in the removal of dummies treated with phenethyl acetate or with the mixture (Table 3).

Discussion

This is the first identification of volatile compounds associated with diseased honeybee larvae that elicit hygienic behavior in adult honeybees. Three volatile compounds collected from larvae infected with the fungal pathogen, *Ascosphaera apis*, were not detected in healthy brood, and elicited electroantennogram responses from adult honeybees. Our bioassays showed that of the three compounds, phenethyl acetate elicited the greatest hygienic responses from bees in field colonies.

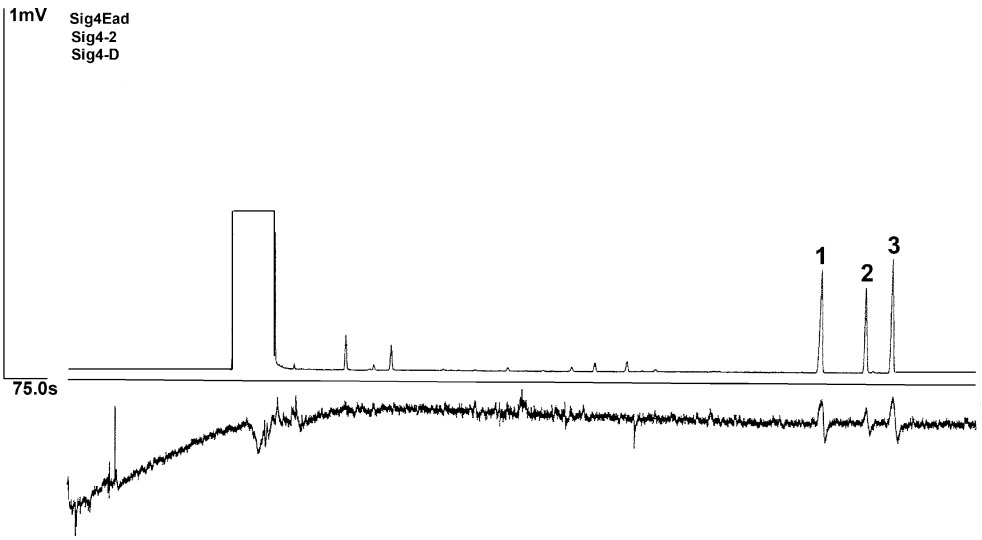

Fig. 2 Representative coupled gas chromatographic-electroantennographic detection (GC-EAD; flame ionization detection response shown on top; EAD response shown on bottom) traces of compounds collected from early stage chalkbrood-infected larvae, using antennae of 15–20 day-old honeybees. Numbered peaks correspond to compounds that elicited consistent EAD responses: 1) phenethyl acetate, 2) 2-phenylethanol, and 3) benzyl alcohol

Table 1 Student's *t* and *p* values of delta contrasts between odorant treatments for proportion of topically treated larvae removed by adult bees at 24 hours. Larvae were treated with 0.05 µl of an individual chemical or a mixture of the three chemicals

$\alpha = 0.05$, $df = 54$.

	Water		Mixture		Benzyl alcohol		Phenyl Ethanol	
	t	*P*	*t*	*P*	*t*	*P*	*t*	*P*
Phenethyl Acetate	8.245	**< 0.001**	5.920	**< 0.001**	11.642	**< 0.001**	8.870	**< 0.001**
2-Phenylethanol	5.549	**< 0.001**	3.280	**0.002**	3.454	**0.001**		
Benzyl alcohol	4.473	**< 0.001**	6.530	**< 0.001**				
Mixture	6.496	**< 0.001**						

Colony-level behavioral responses to parasites and pathogens are an integral part of social immunity, but reports of social animals detecting and responding to diseased conspecifics are not common. Such behavioral responses generally involve avoidance or engagement (e.g., grooming or removal) of conspecifics that are infected, which likely involves the detection of disease-related chemical stimuli. For example, gregarious Caribbean spiny lobsters *Panulirus argus*, which occupy communal dens, exhibit avoidance behavior of lobsters infected with a lethal virus, even during early periods when the lobsters are not yet infectious (Behringer et al., 2006). Leaf cutting ants, *Acromyrmex echinatior,* use self- and allo-grooming behaviors in defense of the fungal disease *Metarhizium anisophae* (Hughes et al., 2002). Ants detect oleic and linoleic acids, emitted from dead adult conspecifics in their nests, and exhibit necrophoric or undertaking behavior, which favor colony health by reducing contact with potential pathogens (Wilson et al 1958; Akino and Yamaoka, 1996; Howard and Tschinkel, 1976). Honeybees also display necrophoric behavior, although the chemical cues have not been identified (Visscher, 1983). Our study demonstrated that disease-related odorants are important because the early detection and efficient removal of the source of the pathogen reduce disease transmission among individuals within the nest.

Our field bioassays confirmed two hypotheses, previously tested only in laboratory assays (Masterman et al., 2001; Spivak et al., 2003): 1) hygienic behavior of honey bees is elicited by olfactory stimuli; and 2) the expression of hygienic behavior depends on the olfactory response threshold of individual bees within the colony. Colonies that contain a majority of bees with high olfactory sensitivity respond quickly to low concentration stimuli associated with diseased brood, while colonies of bees with lower sensitivity take longer to respond, allowing transmission of spores and expression of clinical symptoms.

The compounds that elicited consistent GC-EAD responses, phenethyl acetate, 2-phenylethanol, and benzyl alcohol, were present both in larvae in the early stages of infection and in mummies, but were absent from healthy larvae. At this time, it is unclear whether the chemicals are produced by the larvae (pheromonal) or by the fungus (kairomonal). Laboratory inoculation with chalkbrood of potato dextrose agar, pollen and homogenized and sterilized brood failed to produce emissions of these volatiles, suggesting that the compounds are produced only in the presence of live brood (Baldwyn Torto, USDA, unpublished data).

When 0.5 µl of synthetic disease-associated volatile chemicals were applied topically to healthy larvae, adult bees responded to the olfactory stimulus and removed healthy larvae as though they were diseased. The degree of hygienic behavior, as indicated by the amount of freeze-killed brood removed within 24 h, was affected by the colony level response. The more hygienic a colony, the more likely the removal of larvae treated topically with disease-associated compounds. Bees removed up to 100% of healthy larvae treated with phenethyl acetate within 24 h, which is roughly the same time it takes bees from a rapid-hygienic colony to remove freeze-killed brood. We have not determined the odor of freeze-killed brood for comparison.

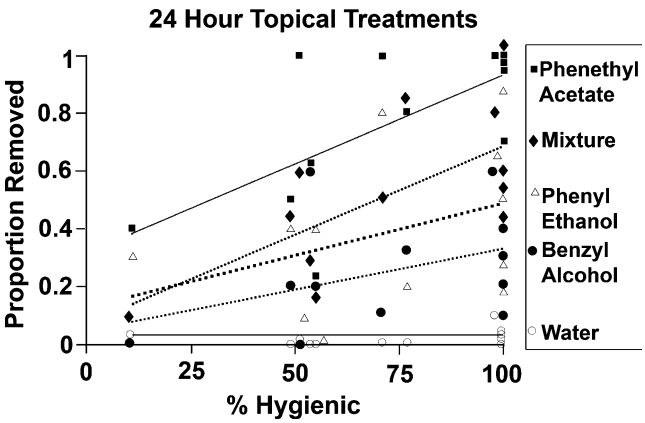

Fig. 3 Regression lines comparing the proportion of healthy larvae removed by adult bees, for each treatment at 24 h (y-axis), to the degree of hygienic behavior [based on the percent of dead brood removed in the freeze-killed brood assay (x-axis)]. Each point corresponds to the results from one of the twelve colonies treated. Treatments applied topically to larvae included de-ionized water (N=10 larvae per colony), phenethyl acetate, 2-phenylethanol, benzyl alcohol, or a mixture of the three chemicals (N=25 larvae per colony for each chemical treatment). Regression lines correspond to the compound listed to the right of each line. Significantly different proportions of larvae were removed for each treatment (Table 1; Student's *t,* a= *0.05, df= 54; P*<0.002)

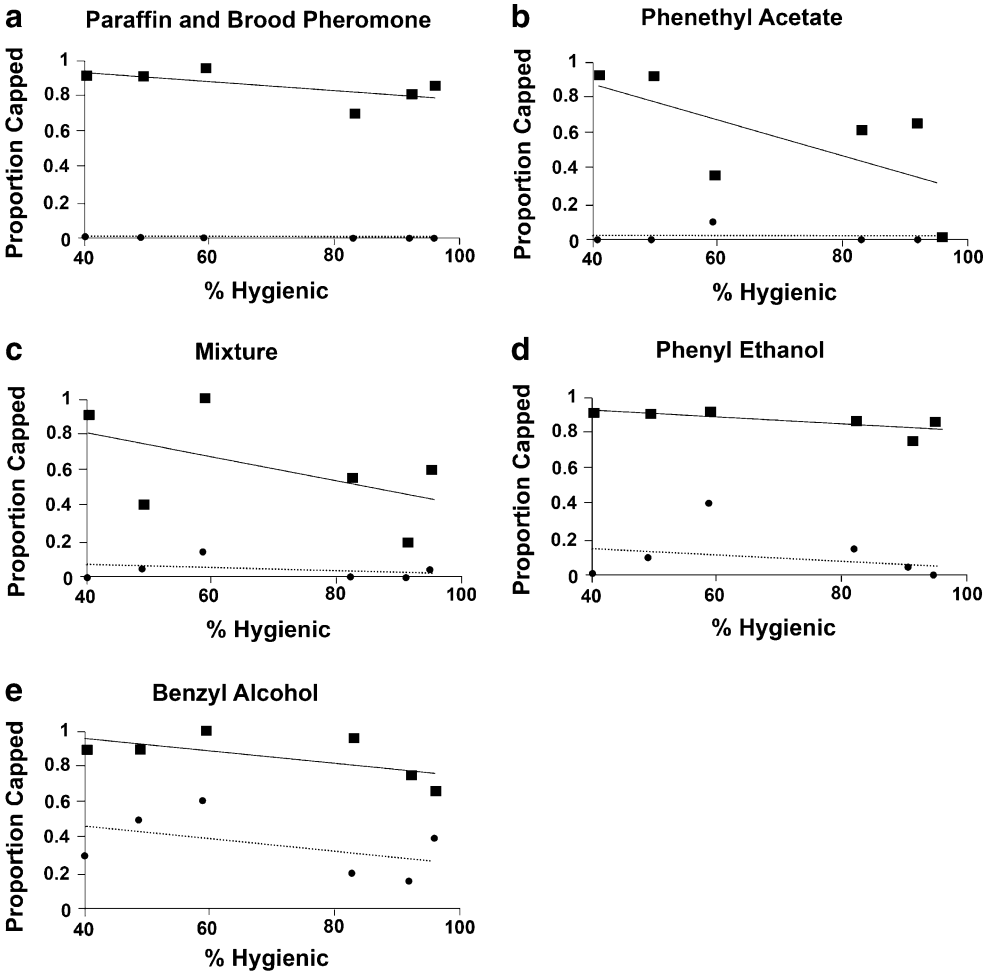

Fig. 4 Regression lines comparing the proportion of paraffin larval dummies capped with wax by adult bees to the degree of hygienic behavior (based on the percent of dead brood removed in the freeze-killed brood assay). Bees are unable to remove paraffin dummies, thus a lower proportion of capping was an indication of rejection of the cell's contents by adult bees; i.e., hygienic behavior. A) Rejection of dummies treated with brood pheromone (squares, top line) and paraffin dummies (circle, bottom line). B – E) Rejection of dummies treated with each compound or a mixture of the compounds, at high concentrations (10^{-2}; circles, bottom lines) and low concentrations (10^{-9}; squares, top lines). Each point corresponds to the results from one of the six colonies treated. Treatments (20 per treatment per colony): paraffin wax only, paraffin wax plus brood pheromone (methyl linolenate), paraffin wax plus brood pheromone plus one of phenethyl acetate, 2- phenylethanol, benzyl alcohol, or a mixture of the three

Bees are very sensitive to abnormal stimuli from larvae. For example, when larvae are touched with a paintbrush, larvae are removed within 5 h (Free and Winder, 1983). Our original intent was to test the response-threshold hypothesis by diluting disease-related compounds in a solvent. However, we observed that application of hexane or pentane alone induced very rapid removal, within 4 h, indicating that solvents elicited an unnatural and abnormally fast removal response. Although bees did not remove larvae treated with water (control in one assay), water was not a suitable solvent for phenethyl acetate, 2-phenylethanol, or benzyl alcohol. Thus we used an indirect bioassay to test

Table 2 Student's *t* and *p* values of delta contrasts between various treatments of paraffin larval dummies capped with wax by adult bees at 24 hours. Paraffin dummies were treated with one component of brood pheromone (methyl linolenate) alone at a 10^{-2} concentration (v/v), or brood pheromone (10^{-2}) plus one additional chemical or a mixture of three chemicals at a 10^{-2} concentration (v/v)

	Brood Pheromone (methyl linolenate)10^{-2}		Paraffin wax		Brood Pheromone+ Mixture 10^{-2}		Brood Pheromone+ Benzyl alcohol 10^{-2}		Brood Pheromone+ 2- Phenylethanol 10^{-2}	
	t	*P*	*t*	*P*	*t*	*P*	*t*	*P*	*t*	*P*
Brood Pheromone+Phenethyl Acetate 10^{-2}	7.753	**< 0.001**	0.700	0.483	1.110	0.278	4.780	**< 0.001**	2.690	**0.013**
Brood Pheromone+2- Phenylethanol 10^{-2}	9.851	**< 0.001**	3.629	**< 0.001**	1.110	**0.049**	4.248	**< 0.001**		
Brood Pheromone+Benzyl alcohol 10^{-2}	7.308	**< 0.001**	5.772	**< 0.001**	5.199	**< 0.001**				
Brood Pheromone+Mixture 10^{-2}	9.359	**< 0.001**	1.957	**0.050**						
Paraffin wax	8.735	**< 0.001**								

$\alpha = 0.05$, $df = 29$.

Table 3 Student's *t* and *p* values of delta contrasts between various treatments of paraffin larval dummies capped with wax by adult bees at 24 hours. Paraffin dummies were treated with one component of brood pheromone (methyl linolenate) alone at a 10^{-2} concentration (v/v), or brood pheromone (10^{-2}) plus one additional chemical or a mixture of the three chemicals at a 10^{-9} concentration

	Brood Pheromone (methyl linolenate) 10^{-2}		Paraffin wax		Brood Pheromone+ Mixture 10^{-9}		Brood Pheromone+ Benzyl alcohol 10^{-9}		Brood Pheromone+ 2-Phenylethanol 10^{-9}	
	t	*P*	*t*	*P*	*t*	*P*	*t*	*P*	*t*	*P*
Brood Pheromone+Phenethyl Acetate 10^{-9}	4.826	< **0.001**	5.160	< **0.001**	0.688	0.497	4.965	< **0.001**	4.965	< **0.001**
Brood Pheromone+2-Phenylethanol 10^{-9}	0.187	0.852	6.650	< **0.001**	4.382	< **0.001**	0.000	1.000		
Brood Pheromone+Benzyl alcohol 10^{-9}	0.187	0.852	6.770	< **0.001**	4.383	< **0.001**				
Brood Pheromone+Mixture 10^{-9}	4.235	< **0.001**	5.353	< **0.001**						
Paraffin wax	6.613	< **0.001**								

*$\alpha = 0.05$, $df = 29$.

the response-threshold hypothesis by combining brood pheromone and disease volatiles at two different concentrations in paraffin larval dummies. When one component of brood pheromone, methyl linolenate, was added to a paraffin dummy placed within a cell, bees capped that dummy with wax as though it were a 5th instar ready to pupate. However, when both brood pheromone and disease-related volatile(s) were added to the paraffin, bees from rapid-hygienic colonies did not cap the dummies with wax; apparently they detected the disease-associated volatiles over the brood pheromone and behaved accordingly. We observed that bees with less olfactory sensitivity (slow-hygienic colonies) were not able to detect the disease-associated volatiles over the brood pheromone, and initiated the capping of the dummy. We determined that there was a significant correlation between the level of hygienic behavior, as indicated by the freeze-killed brood assay and the lack of capping of the treated paraffin dummies. Thus, the lack of capping in this assay was an indirect, but sensitive, measure of the level of hygienic behavior of the tested colonies.

We expected that a mixture of phenethyl acetate, 2-phenylethanol, and benzyl alcohol would elicit an increased response from honeybees over any of the compounds tested singly. However, our two bioassays showed no difference between responses to the mixture and that of phenethyl acetate alone, suggesting a lack of mixture-specific synergistic effects. It should be noted that only one ratio of components was tested in our bioassays; it is possible that a different ratio may give enhanced stimulation.

The results of this study have a practical application for beekeepers and important implications for the health of honey bees. It is critical that the beekeeping industry reduce its reliance on chemical treatments for diseases and parasitic mites due to the risk of contaminating hive products with residue, and the development of resistance to the treatments by the pathogens and parasites (Milani, 1999; Wallner, 1999). The most sustainable solution is to breed bees selectively for resistance to diseases and mites. To date, hygienic behavior is one of the few resistance mechanisms that has a simple field assay that beekeepers can employ. Colonies that rapidly remove freeze-killed brood tend to be behaviorally resistant to two brood pathogens, chalkbrood, *A. apis*, and American foulbrood, *Paenibacullus larvae* (Spivak and Reuter, 2001a). Colonies that rapidly remove freeze-killed brood also tend to detect and remove pupae infested with the parasitic mite, *Varroa destructor* (Spivak, 1996). However, the correspondence between the removal of freeze-killed brood and removal of diseased or parasitized brood is not perfect. For example, when 18 rapid-hygienic colonies, as determined by the freeze-killed brood assay, were challenged with American foulbrood pathogen, two colonies developed persistent clinical symptoms of the disease (Spivak and Reuter, 2001a). Also, colonies selected for hygienic behavior still require treatments to control *V. destructor*, as the bees are unable to detect and remove sufficient infested pupae during high mite infestations (Spivak and Reuter, 2001b; Ibrahim et al, 2007). While hygienic behavior is a generalized response to the presence of diseased, parasitized, and abnormal pupae, it is necessary to have a more specific assay to breed colonies selectively for resistance to a particular pathogen or parasite. The experiments performed here could be modified to focus on the specific stimuli that elicit detection and removal of larvae infected with the American foulbrood pathogen and pupae infested with *V. destructor*, thus furthering efforts to breed bees for resistance to these economically important problems.

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2003; Billen 2006). The insect body is covered by a cuticle that is made up of layers, the outermost layer (epicuticle) is composed of a mixture of lipids synthesized by oenocytes (Lockey 1988). These lipids regulate permeability and prevent desiccation (Ayasse et al. 2001; Ayasse 2006). However, there is evidence that the epicuticlar lipids of Hymenoptera also act as pheromones and/or as caste/sex recognition cues (Singer 1998; Ayasse et al. 2001; Ayasse 2006). Bioassays have demonstrated that cuticular hydrocarbons of some species act as sex pheromones and that the cuticular hydrocarbons of some parasitic insects are similar to those of their respective host species (Singer 1998). As an example, cuticular waxes of drones, food storers, foragers, and queen attenders of *Apis mellifera* are used to discriminate intraspecific roles inside the colony (Fröhlich et al. 2001). In contrast, behavioral and chemical assays that involve males and females of the solitary bee *Amegilla dawsoni* suggest that cuticular hydrocarbons are important in sexual communication (Simmons et al. 2003). Cuticular hydrocarbons also are a clue to understanding one of the most spectacular biological phenomena: orchid pollination through sexual mimicry (or pseudocopulation) (Ayasse 2006). Sexual mimicry was detected in the Neotropical orchids *Trigonidium obtusum* and *Mormolyca ringens*, which are pollinated by *Plebeia droryana* males and *Nannotrigona testaceicornis* stingless bees, respectively (Singer 2002; Singer et al. 2004).

The Dufour gland is important in bees. It is located at the base of the sting apparatus, ventrally to the poison gland, and opens into the dorsal vaginal wall (Roubik 1989; Cruz-López et al. 2001; Abdalla et al. 2004). It normally is present in some females of Apoidea, but is absent or vestigial in workers of *Melipona* species (Patricio et al. 2003). The Dufour glands secrete a variety of compounds (macrolactones, hydrocarbons, triglycerides, and terpenoids), and each species has a particular chemical composition (Abdalla et al. 2004). In *Nannotrigona testaceicornis* workers, the major substance is geranylgeranyl acetate (Cruz-López et al. 2001); the same compound is detected in labial secretions of *Bombus* males and used as a territorial pheromone (Kullenberg et al. 1970). In *Melipona bicolor* virgin queens, the Dufour gland contains predominantly hydrocarbons, while in *Melipona bicolor* physogastric queens, isobutyrate and acetate esters are the main components. Compositional differences in the Dufour gland of queens may indicate the queen's fecundity condition, while the oxygenated compounds in secretions of physogastric queens may increase their attractiveness to males (Abdalla et al. 2004).

Communication between bees also can be achieved through cephalic volatile compounds. Many species of stingless bees and honeybees produce cephalic substances that serve as alarm or sexual pheromones, and odor trail markers (Luby et al. 1973; Hefetz et al. 1979; Bian et al. 1984; Engels et al. 1987, 1997; Roubik et al. 1987; Francke et al. 2000; Cruz-López et al. 2005). *Trigona, Oxytrigona, Scaptotrigona, Cephalotrigona, Lestrimelitta*, and *Nannotrigona* deposit cephalic substances on vegetation as a trail marker (Roubik 1989). Analyses of *Frieseomelitta varia* and *F. silvestrii* volatiles in cephalic secretions reveal the presence of volatile oxygenated compounds (Cruz-López et al. 2002). Cephalic secretions from newly emerged, nurse, and forager workers, virgin and physogastric queens, and males of *Melipona bicolor* are caste-sex specific. They consist of hydrocarbons, alcohols, esters, and acids. These secretions differentiate the tasks performed by workers and the physiological reproductive condition of queens (Gracioli-Vitti et al. 2004).

Both species studied in our present work, *Nannotrigona testaceicornis* and *Plebeia droryana*, belong to the Trigonini group. *P. droryana* is one species in a large group of small stingless bees, the *Plebeia* group, and is considered "basic" since it does not have many derived characters. Members of the *Plebeia* group are found in the New World, as well as in Australia, Asia, and Africa (Moure 1961; Michener 2000). The chemical features of caste-sex recognition in *N. testaceicornis* and *P. droryana* (Meliponinae) are limited, and studies of interspecific and intraspecific communication are necessary to understand the intricate behaviors.

Methods and Materials

Biological Material All stingless bees were collected at the "Aretuzina" farm in São Simão, SP, Brazil. Foraging workers from three different *N. testaceicornis* colonies and one colony of *P. droryana* (N=8 of each species) were collected while flying away from their colonies. Males of *P. droryana* (N=8) were collected while attempting copulation with *Trigonidium obtusum* flowers (Orchidaceae) (Singer 2002). Males of *N. testaceicornis* (N=8) were collected while attempting copulation with *Mormolyca ringens* flowers (Orchidaceae) (Singer et al. 2004).

Chemicals *n*-Alkane standards (C_{10}–C_{37}) were obtained from petroleum (100 ml), by silica gel (Acros Organics, USA, 0.035–0.070 mm, 60 A) column chromatography using bidistilled hexane as solvent. The distilled and bidistilled solvents—ethyl acetate (99.5%) and hexane (98.5%) both from Synth, Brazil—and diethyl ether (99%); Nuclear, Brazil) were obtained according to the method described by Perrin et al. (1980). Iodine (Rothyl, USA, 99%), dimethyl disulfide (Aldrich, Germany, 99%), anhydrous magnesium sulphate (Nuclear, Brazil, 99%), anhydrous sodium thiosulphate (Merck, Brazil, 99%),

chloroform-D (Cambridge Isotope Laboratories, USA, 99.8%), farnesol—mixture of isomers (Aldrich, USA, 95%), farnesyl acetate—mixture of isomers (Aldrich, USA, 95%), (*S*)-(+)-2-heptanol (Aldrich, USA, 99%), (*S*)-(+)-2-nonanol (Aldrich, USA, 97%), (±)-2-heptanol (SAFC, USA, 97%), (±)-2-nonanol (Aldrich, USA, 99%), and (±)-2-undecanol (Fluka, France, 98%) were used as provided. Synthetic standards of lauryl acetate, octadecyl acetate, tetradecyl dodecanoate, and hexadecyl hexadecanoate—all 99% purity—were prepared in our laboratory using usual procedures (Larock 1989). The spectroscopic properties of these synthetic standards were compared with data in the literature (Bertsch et al. 2004).

Cuticular Wax Extracts Foraging workers (*N*=8) and males (*N*=8) of *N. testaceicornis* and *P. droryana* were freeze killed (−18°C) and dipped into bidistilled hexane (2 ml, for 5 min). The solvent was evaporated under a flow of nitrogen. Extracts were dissolved in hexane (1 mg/ml) and analyzed by gas chromatography-mass spectrometry (GC-MS) by injecting 1 µl of each extract (Table 1).

Abdomen and Cephalic Extracts After washing with bidistilled hexane (2 ml), bee abdomens and heads of foraging workers and males of *N. testaceicornis* and *P. droryana* were separated and macerated with bidistilled ethyl acetate (2 ml). Extracts were filtered, evaporated with nitrogen, dissolved in ethyl acetate (1 mg/ml), and analyzed by GC-MS (1 µl of each extract) (Table 1).

GC-MS Analyses were carried out with an HP 6890/5973 system equipped with an HP5 fused silica capillary column (30 m×0.25 mm×0.25 µm). Helium was used as carrier gas, at a flow rate of 1 ml/min in splitless mode. Spectra were taken at 70 eV, and the scanning speed was 2.89 scans/s from *m/z* 40 to 550. The interface temperature was maintained at 280°C, and the injector temperature was 250°C. The temperature program used to analyze wax samples went from 50°C–290°C at 12°C/min with a final hold time of 20 min. Derivatized samples used a temperature program from 50°C–290°C at 12°C/min with a final hold time of 50 min.

Chiral GC-FID Analyses GC-FID analyses were conducted with an Agilent Technologies 6850 chromatograph equipped with a chiral capillary column (Chrompack CP-Chirasil-Dex CB; 25 m×0.25 mm×0.25 µm). The carrier gas was highly purified hydrogen with a flow rate of 0.8 ml/min. Injector and detector temperatures were 180°C and 220°C, respectively. (*S*)-(+)-2-Nonanol and (±)-2-nonanol were dissolved in bidistilled ethyl acetate (100 µg/ml) and injected in split mode (1/50); the oven temperature was programmed from 50°C to 100°C at 0.4°C/min. The same procedure was used to analyze a 2:1 mixture of (±)-2-nonanol (100 µg/ml) and (*S*)-2-nonanol (50 µg/ml). Abdominal and cephalic extracts of *P. droryana* males were dissolved in 20 µl of bidistilled ethyl acetate. A 4:1 mixture composed of male *P. droryana* extracts and (±)-2-nonanol (25 µg/ml) were co-injected in splitless mode. The temperature program increased from 50°C–100°C at 0.4°C/min, then from 100°C–180°C at 30°C/min), and then held at 180°C for 20 min.

Retention Indexes Identification of the extract components were made based on comparison of their retention indices (Van den Dool and Kratz 1963), as well as by computer matching of the mass spectra obtained with those in the Wiley 275 mass spectral library of the GC/MS data system and other published mass spectra (Adams 2001; Table 1). Retention indexes for the *n*-alkane standards (C_{10}–C_{37}) were taken as whole numbers from 1000 to 3700. The (*Z*) and (*E*)-alkenes were identified by their Equivalent Chain Length (ECL = RI/100) values compared with the literature (Ashes et al. 1980; Howard and Baker 2003).

DMDS/I_2 Derivatizations The samples with alkenes were dissolved in bidistilled hexane (2 ml) and treated with dimethyl disulfide (DMDS; 200 µl) and iodine solution (100 µl of 32 mg of I_2 in 2 ml of distilled diethyl ether). Reaction mixtures were stirred overnight at 50°C with magnetic stirring. Reaction mixtures were quenched with aqueous sodium thiosulfate solution (2 ml of 1 g $Na_2S_2O_3$ in 10 ml of distilled water). The organic phase was extracted, dried over anhydrous MgSO4, and evaporated to dryness with a nitrogen stream (Buser et al. 1983; Vincenti et al. 1987). The derivativitized samples were dissolved in bidistilled hexane (50 µl), and 1 µl of each sample was analyzed by GC-MS.

NMR Analyses ^{1}H-NMR spectra were recorded with Varian INOVA 500 (499.88 MHz) spectrometer equipped with a 5 mm probe. $CDCl_3$ was used as the solvent. ^{13}C- NMR analyses were recorded with a Varian INOVA 500 (125.69 MHz) spectrometer equipped with a nanoprobe of 50 µl. $CDCl_3$ (77.0 ppm) was used as internal standard. Methyl, methylene, methane, and carbon nonbonded to hydrogen were discriminated using DEPT-135° and DEPT-90° spectra (distortionless enhancement by polarization transfer).

Field Tests The responses of *P. droryana* and *N. testaceicornis* to the synthetic secondary alcohols [(*S*)-(+)-2-heptanol, (*S*)-(+)-2-nonanol, a 1:1 mixture of (*S*)-(+)-2-heptanol and (*S*)-(+)-2-nonanol, (±)-2-heptanol, (±)-2-nonanol, and (±)-2-undecanol] were evaluated in December 2008 and again in January 2009. Compounds were tested in an open

Table 1 GC-MS analyses of *Nannotrigona testaceicornis* and *Plebeia droryana* extracts

Numbers	Compounds	t_R (min)	RI (calc.)	RI (lit.)	NANNOTRIGONA TESTACEICORNIS						PLEBEIA DRORYANA					
					MALES			WORKERS			MALES			WORKERS		
					Cuticular Waxes (%)[a]	Abdominal extract (%)[a]	Cephalic extract (%)[a]	Cuticular Waxes (%)[a,b]	Abdominal extract (%)[a,b]	Cephalic extract (%)[a,b]	Cuticular Waxes (%)[a]	Abdominal extract (%)[a]	Cephalic extract (%)[a]	Cuticular Waxes (%)[a]	Abdominal extract (%)[a]	Cephalic extract (%)[a]
Hydrocarbons																
1	Pentadecene[c]	10.78	1482	–	–	–	–	–	–	0.72± 0.26	–	–	–	–	–	–
2	Pentadecane	10.97	1500	1500	–	–	–	0.38± 0.53	–	1.30± 0.04	–	–	–	–	–	–
3	2-phenyl-tridecane	14.87	1916	–	5.18	–	–	–	–	–	–	–	–	–	–	–
4	Heneicosane	16.36	2100	2100	–	–	–	–	–	0.08± 0.11	–	–	–	–	–	–
5	9-tricosene	17.81	2295	–	–	–	–	–	–	1.72± 0.59	–	–	–	–	–	–
6	Tricosane	17.85	2300	2300	1.46	–	1.16	1.83± 0.23	–	1.17± 0.07	–	–	–	–	–	–
7	(*Z*)-9-pentacosene	19.07	2476	–	–	–	–	1.57± 0.37	–	0.60± 0.47	2.03	1.14	2.97	1.58	0.14	2.10
8	Pentacosane	19.23	2500	2500	4.65	2.05	1.76	5.73± 1.29	0.24±0.07	1.63± 0.14	8.74	1.83	3.16	10.73	0.42	5.58
9	(*Z*)-9-hexacosene	19.73	2579	–	–	–	–	–	–	–	1.00	0.48	1.04	0.71	0.34	0.89
10	(*Z*)-9-heptacosene	20.38	2676	–	2.50	1.83	1.99	3.59± 1.53	0.26±0.00	2.51± 0.46	49.09	16.25	40.17	41.66	2.88	48.47
11	Heptacosane	20.54	2700	–	8.57	4.02	1.96	12.25± 1.00	0.28±0.04	1.48± 0.35	6.00	0.89	1.23	4.98	0.21	2.36
12	(*Z*)-9-octacosene	21.10	2776	–	–	–	0.79	0.83± 1.17	0.07±0.01	0.78± 0.11	0.42	0.16	–	0.31	–	0.27
13	(*Z*)-9-nonacosene	21.94	2879	–	45.26	56.17	46.80	40.65± 1.53	2.97±0.40	28.52± 3.79	10.33	2.92	5.92	12.58	0.78	12.30
14	Nonacosane	22.12	2900	–	1.90	–	–	2.30± 0.29	–	1.20± 1.70	3.21	0.52	0.47	1.10	–	0.53
15	9, 21-hentriacontadiene	23.75	3053	–	4.18	7.08	6.17	2.62± 1.04	0.33±0.04	4.36± 0.47	–	–	–	–	–	–
16	(*Z*)-9-hentriacontene	24.05	3078	–	5.63	5.88	5.90	3.73± 0.20	0.28±0.05	3.19± 0.99	8.24	1.96	4.34	10.40	0.63	11.42
17	Hentriacontane	24.31	3100	–	–	–	–	–	–	–	1.66	0.27	–	–	–	–
18	9-tritriacontane	27.13	3278	–	–	–	–	–	–	–	1.72	0.65	1.52	2.38	0.17	2.82
Terpenes																
19	α-copaene	9.76	1387	1376	1.09	2.19	–	1.38± 0.50	–	0.26± 0.12	–	–	–	0.33	0.04	0.22

20	*Ar*-curcumene	10.83	1487	1483	1.30	1.80	–	1.11± 0.01	–	–	–	–	–	0.17	–	0.17
21	α-murolene	11.07	1510	1499	0.01	–	–	0.76± 0.09	–	–	–	–	–	–	–	–
22	δ-cadinene	11.27	1529	1524	4.41	4.26	–	4.46± 0.30	0.01±0.01	–	0.37	–	–	1.08	0.10	0.63
23	1- *cis*- calamenene	11.32	1534	1521	1.21	1.29	–	1.31± 0.12	–	–	–	–	–	0.23	–	0.14
24	α-calacorene	11.55	1557	1542	–	–	–	0.25± 0.35	–	–	–	–	–	–	–	–
25	Cadalene	12.85	1690	1674	1.34	–	–	0.29± 0.41	–	–	–	–	–	–	–	–
26	(*E*, *E*)-farnesol[e]	13.18	1724	1722	–	–	–	–	–	–	–	–	–	–	25.72	–
27	(*E*, *Z*)-farnesol[e]	13.35	1743	1742	–	–	–	–	–	–	–	–	–	–	0.17	–
28	7,11,15-trimethyl-3-methylene-1,6,10,14-hexadecatetraene	14.86	1914	–	–	–	–	–	0.37±0.04	0.68± 0.17	–	–	–	–	–	–
29	Geranyl linalool	17.06	2191	–	–	–	–	–	–	–	–	–	–	0.64	10.39	–
30	Squalene	21.41	2815	–	1.64	2.06	–	0.57± 0.80	–	–	–	0.17	–	1.43	–	–
Terpenic Esters																
31	Nerolidyl acetate	13.13	1719	1714	–	–	–	–	–	–	–	–	–	2.30	–	–
32	Farnesyl acetate[e]	14.16	1834	1818	–	–	–	–	0.45±0.09	–	–	–	–	–	–	–
33	Geranyl-geranyl acetate[f]	17.91	2308	–	–	–	–	–	83.90± 0.58	–	–	–	–	–	–	–
Acids																
34	Dodecanoic acid	11.57	1557	–	–	–	–	–	–	–	–	0.16	–	–	–	–
35	Tetradecanoic acid	13.50	1759	–	–	–	–	–	–	–	–	0.48	0.33	–	0.16	–
36	Hexadecanoic acid	15.27	1963	–	–	–	–	–	–	–	–	2.56	0.84	–	0.09	–
37	Linoleic acid and linolenic acid[d]	16.83	–	–	–	–	–	–	–	–	–	46.59	–	–	–	–
38	Octadecanoic acid	16.93	2174	–	–	–	–	–	–	–	–	5.61	–	–	–	–
Esters																
39	Lauryl acetate[e]	12.05	1606	–	–	–	–	–	0.04±0.04	–	–	–	–	–	–	–
40	Octadecyl acetate[e]	17.19	2208	2210	–	–	–	–	–	–	–	–	0.66	–	–	–
41	Tetradecyl dodecanoate[e]	21.03	2767	–	–	–	–	–	–	–	–	–	–	–	0.27	–
42	Hexadecyl hexadecenoate	28.58	3349	–	–	–	7.79	–	–	5.86± 2.47	–	–	–	–	–	–
43	Hexadecyl hexadecanoate[e]	28.83	3360	–	–	–	–	–	–	0.48± 0.68	–	–	–	–	–	–
44	Hexadecenyl octadecenoate	33.59	3538	–	–	–	–	–	–	2.47± 1.58	–	–	–	–	–	3.59
45	Hexadecyl octadecenoate	33.99	3550	–	–	–	6.28	–	–	14.56± 3.32	–	–	–	–	–	–

Table 1 (continued)

Numbers	Compounds	t_R (min)	RI (calc.)	RI (lit.)	Nannotrigona testaceicornis						Plebeia droryana					
					MALES			WORKERS			MALES			WORKERS		
					Cuticular Waxes (%)[a]	Abdominal extract (%)[a]	Cephalic extract (%)[a]	Cuticular Waxes (%)[a,b]	Abdominal extract (%)[a,b]	Cephalic extract (%)[a,b]	Cuticular Waxes (%)[a]	Abdominal extract (%)[a]	Cephalic extract (%)[a]	Cuticular Waxes (%)[a]	Abdominal extract (%)[a]	Cephalic extract (%)[a]
Spiroacetal																
46	(*E,E*)-2,8-dimethyl-1,7-dioxaspiro[5.5] undecane	6.85	1142	–	–	–	0.58	–	–	2.36± 1.67	–	–	–	–	–	–
Alcohol																
47	(*S*)-2-nonanol[e]	6.34	1100	1098	–	–	–	–	–	–	–	0.08	0.40	–	–	–
Aldehyde																
48	Nonanal	6.39	1105	1102	–	–	–	–	–	0.71± 0.38	–	0.15	–	–	–	–
49	Tetradecanal	12.21	1620	1613	–	–	–	–	–	–	–	–	–	–	46.82	–
50	Hexadecanal	14.05	1820	–	–	–	–	–	–	–	–	–	–	–	3.43	–
Steroids																
51	Ergostadien-3β-ol	26.22	3227	–	–	–	–	–	–	–	–	1.95	9.66	–	0.38	4.40
52	Ergost-5-en-3β-ol	26.34	3233	–	–	–	8.32	–	–	4.76± 1.41	–	1.68	5.53	–	0.01	1.00
53	Stigmast-5-en-3β-ol	27.84	3315	–	–	3.04	2.60	–	0.27±0.04	2.57± 0.47	–	2.62	2.79	–	0.71	0.61
54	Stigmastadien-3β-ol	28.15	3329	–	–	–	–	–	–	–	–	4.34	3.92	–	1.22	0.86
Unknown compounds					9.67	8.33	7.90	14.45	10.56	16.06	7.19	6.54	15.05	7.39	4.92	5.23
Total					100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

There are N=8 for each sample. The quantities of extracts from each individual male and worker bee: *N. testaceicornis* cuticular waxes −0.100 mg/ male and 0.071 mg/ worker; *P. droryana* cuticular waxes −0.113 mg/ male and 0.100 mg/ worker; *N. testaceicornis* abdominal extract −0.050 mg/ male and 0.058 mg/ worker; *P. droryana* abdominal extract −0.088 mg/ male and 0.050 mg/ worker; *N. testaceicornis* cephalic extract −0.050 mg/ male and 0.029 mg/ worker; *P. droryana* cephalic extract −0.075 mg/ male and 0.025 mg/ worker

t_R Retention times, *RI (calc.)* calculated retention indexes, *RI (lit.)* retention indexes taken from literature (Adams 2001),-not found

[a] Relative abundances (%) and tentative identification using mass spectra and retention indeces comparison

[b] Data are shown the mean ± SD of the *N. testaceicornis* foraging workers of three colonies

[c] The double bond position was not determinated for this alkene

[d] Linoleic acid and linolenic acid co-eluted in one only peak

[e] Synthetic standards were used to confirm these structures

[f] This compound was identified by ^{1}H NMR

field close to several *Meliponini* nests at the University of São Paulo, Ribeirão Preto-SP, Brazil. Strips of filter paper containing approximately 2 μl (1.65 mg) of each (*S*)-2-alkanol were deposited 1.5 m from a shelf containing colonies of *P. droryana*, *N. testaceicornis*, *S.* aff. *depilis*, *F. varia*, *F. silvestrii, Tetragonisca angustula* and *Friesella schrottkyi*. The experiment was repeated on three different shelves containing the same species. Strips were monitored for 10 min to test the ability of alkanols to elicit behavioral responses. At the end of the observing period, the specimens around the paper trips were collected, counted and identified.

Results

Extracts of Cuticular Waxes N. testaceicornis male cuticular waxes (eight specimens=0.800 mg) were found to be composed of *n*-alkanes (16.58%; *n*-C23, *n*-C25, *n*-C27, and *n*-C29), *n*-alkenes (53.39%; *n*-(*Z*)-9-C27, *n*-(*Z*)-9-C29, and *n*-(*Z*)-9-C31), an *n*-alkadiene (4.18%; *n*-9,21-C31), an aromatic alkane (5.18%; 2-phenyl-tridecane), and terpenes (11.0%; α-copaene, *Ar*-curcumene, α-murolene, δ-cadinene, 1-*cis*-calamenene, cadalene, and squalene) (Table 1). *N. testaceicornis* worker cuticular waxes (eight specimens) =0.568 mg) were composed of *n*-alkanes (22.69%; *n*-C15, *n*-C21, *n*-C23, *n*-C25, *n*-C27, and *n*-C29), *n*-alkenes (50.37%; *n*-(*Z*)-9-C25, *n*-(*Z*)-9-C27, *n*-(*Z*)-9-C28, *n*-(*Z*)-9-C29, and *n*-(*Z*)-9-C31), an *n*-alkadiene (2.62%; *n*-9, 21-C31), and terpenes (10.13%; α-copaene, *Ar*-curcumene, α-murolene, δ-cadinene, 1-*cis*-calamenene, α-calacorene, cadalene, and squalene).

P. droryana male cuticular waxes (eight specimens= 0.904 mg) consisted of *n*-alkanes (19.61%; *n*-C25, *n*-C27, *n*-C29, and *n*-C31), *n*-alkenes (72.83%; *n*-(*Z*)-9-C25 to *n*-(*Z*)-9-C29, *n*-(*Z*)-9-C31, and *n*-9-C33), and terpenes (0.37%; δ-cadinene) (Table 1). *P. droryana* foraging worker waxes (eight specimens=0.800 mg) contained *n*-alkanes (16.81%; *n*-C25, *n*-C27, and *n*-C29), *n*-alkenes (69.62%; *n*-(*Z*)-9-C25 to *n*-(*Z*)-9-C29, *n*-(*Z*)-9-C31, and *n*-9-C33), terpenes (3.88%; α-copaene, *Ar*-curcumene, δ-cadinene, 1-*cis*-calamenene, geranyl linalool, and squalene), and a terpenic ester (2.30%, nerolidol acetate).

Abdomen Extracts N. testaceicornis male abdomens (eight specimens=0.400 mg) were composed of *n*-alkanes (6.07%; *n*-C25 and *n*-C27), *n*-alkenes (63.88%; *n*-(*Z*)-9-C27, *n*-(*Z*)-9-C29, and *n*-(*Z*)-9-C31), an *n*-alkadiene (7.08%; *n*-9,21-C31), terpenes (11.60%, α-copaene, *Ar*-curcumene, δ-cadinene, 1-*cis*-calamenene, and squalene), and a steroid (3.04%; stigmast-5-en-3β-ol) (Table 1). The abdomens of *N. testaceicornis* foraging workers (eight specimens=0.464 mg) contained *n*-alkanes (0.52%; *n*-C25 and *n*-C27), *n*-alkenes (3.58%; *n*-(*Z*)-9-C27, *n*-(*Z*)-9-C28, *n*-(*Z*)-9-C29, and *n*-(*Z*)-9-C31), an *n*-alkadiene (0.33%; *n*-9,21-C31), a branched alkatetraene (0.37%; 7,11,15-trimethyl-3-methylene-1,6,10,14-hexadecatetraene), terpenic esters (84.35%; farnesyl acetate and geranyl-geranyl acetate), a long chain ester (0.04%, lauryl acetate), and a steroid (0.27%; stigmast-5-en-3β-ol).

The structure of geranylgeranyl acetate (**1**) was determined by GC-MS and ^{1}H- and ^{13}C-NMR using the crude abdominal secretion extracts of *N. testaceicornis* workers. EIMS (70 eV)–*m/z* (%): 332 (M^{+}, 0.1), 272 (4.0), 257 (4.0), 243 (1.0), 229 (4.0), 217 (1.0), 203 (7.0), 189 (8.0), 175 (4.0), 161 (15.0), 147 (14.0), 133 (19.0), 119 (26.0), 107 (37.0), 93 (56.0), 81 (52.0), 69 (100), 55 (15), 41 (44). ^{1}H NMR (499.88 MHz, $CDCl_3$): δ 1.57–1.70 ppm (4 *s*, 15 H), 1.98 (*m*, 6H), 2.04 (*s*, 3H, CH_3COO), 2.05 (*m*, 6H), 4.60 (*d*, 2H, *J*=6.5 Hz), 5.10 (*m*, 3H), 5.30 (*t*, 1H, *J*=6.5 Hz). ^{13}C NMR (DEPT 135° and 90°, 125.69 MHz, $CDCl_3$): δ 16.00 ppm (CH_3), 16.10 (CH_3), 16.50 (CH_3), 17.70 (CH_3), 21.10 (CH_3), 25.71 (CH_3), 26.22 (CH_2), 26.64 (CH_2), 26.77 (CH_2), 39.55 (CH_2), 39.69 (CH_2), 39.74 (CH_2), 61.42 (CH_2), 118.24 (CH), 123.61 (CH), 124.19 (CH), 124.40 (CH), 135.00 (C), 135.51 (2C), 144.50 (C), 172.50 (C = O).

Abdomens from male *P. droryana* (eight specimens= 0.704 mg) were composed of *n*-alkanes (3.51%; *n*-C25, *n*-C27, *n*-C29, and *n*-C31), *n*-alkenes (23.56%; *n*-(*Z*)-9-C25, *n*-(*Z*)-9-C29, *n*-(*Z*)-9-C31, and *n*-9-C33), a terpene (0.17%; squalene), an alcohol (0.08%; (*S*)-2-nonanol), an aldehyde (0.15%; nonanal), acids (8.81%; *n*-C12, *n*-C14, *n*-C16, *n*-C18, and mixtures of C18:2 and C18:3 fatty acids), and steroids (10.59%; ergostadien-3β-ol, ergost-5-en-3β-ol, stigmast-5-en-3β-ol, and stigmastadien-3β-ol) (Table 1). *P. droryana* foraging worker abdomens (eight specimens= 0.400 mg) contained *n*-alkanes (0.63%; *n*-C25 and *n*-C27), *n*-alkenes (4.94%; *n*-(*Z*)-9-C25, *n*-(*Z*)-9-C27, *n*-(*Z*)-9-C29, *n*-(*Z*)-9-C31, and *n*-9-C33), terpenes (36.42%; mainly terpenic alcohols: (*E*,*E*)-farnesol, (*E*,*Z*)-farnesol, and geranyl linalool), an ester (0.27%; tetradecyl dodecanoate), aldehydes (50.25%; *n*-C14 and *n*-C16), acids (0.25%; *n*-C14 and *n*-C16), and steroids (2.32%; ergostadien-3β-ol, ergost-5-en-3β-ol, stigmast-5-en-3β-ol, and stigmastadien-3β-ol).

Cephalic Extracts of N. testaceicornis males (eight specimens=0.400 mg) were composed of *n*-alkanes (4.88%; *n*-C23, *n*-C25, and *n*-C27), *n*-alkenes (55.48%; *n*-(*Z*)-9-C27, *n*-(*Z*)-9-C29, and *n*-(*Z*)-9-C31), an *n*-alkadiene (6.17%; *n*-9,21-C31), esters (14.07%; mainly constituted of hexadecyl hexadecenoate and hexadecyl octadecenoate), a spiroacetal (0.58%; (*E,E)*-2,8-dimethyl-1,7-dioxaspiro[5.5] undecane) (Kitching et al. 1989; Booth et al. 2006), and steroids (10.92%; ergost-5-en-3β-ol and stigmast-5-en-3β-

ol) (Table 1). *N. testaceicornis* foraging worker cephalic extracts (eight specimens=0.232 mg) were composed of *n*-alkanes (6.86%; *n*-C15, *n*-C23, *n*-C25, *n*-C27, and *n*-C29), *n*-alkenes (38.04%; *n*-9-C15, *n*-9-C23, *n*-(*Z*)-9-C25, *n*-(*Z*)-9-C27, *n*-(*Z*)-9-C28, *n*-(*Z*)-9-C29, and *n*-(*Z*)-9-C31), an *n*-alkadiene (4.36%; *n*-9,21-C31), a branched alkatetraene (0.68%, 7,11,15-trimethyl-3-methylene-hexadecatetraene), a terpene (0.26%; α-copaene), esters (23.37%; hexadecyl hexadecenoate, hexadecyl hexadecanoate, hexadecenyl octadecenoate, and hexadecyl octadecenoate), an aldehyde (0.71%; nonanal), a spiroacetal (2.36%; (*E,E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane) (Booth et al. 2006; Kitching et al. 1989), and steroids (7.33%; ergost-5-en-3β-ol and stigmast-5-en-3β-ol).

P. droryana male cephalic extracts (eight specimens=0.600 mg) contained *n*-alkanes (4.86%; *n*-C25, *n*-C27, and *n*-C29), *n*-alkenes (55.96%; *n*-(*Z*)-9-C25, *n*-(*Z*)-9-C27, *n*-(*Z*)-9-C29, *n*-(*Z*)-9-C31, and *n*-9-C33), acids (1.17%; *n*-C14 and *n*-C16), an ester (0.66%, octadecyl acetate), an alcohol (0.40%, (*S*)-2-nonanol), and steroids (21.90%; ergostadien-3β-ol, ergost-5-en-3β-ol, stigmast-5-en-3β-ol, and stigmastadien-3β-ol) (Table 1). *P. droryana* foraging worker cephalic extracts (eight specimens=0.200 mg) were composed of *n*-alkanes (8.47%; *n*-C25, *n*-C27, and *n*-C29), *n*-alkenes (78.27%; *n*-(*Z*)-9-C25, *n*-(*Z*)-9-C29, *n*-(*Z*)-9-C31, and *n*-9-C33), terpenes (1.16%; α-copaene, *Ar*-curcumene, δ-cadinene, and 1-*cis*-calamenene), and steroids (6.87%; ergostadien-3β-ol, ergost-5-en-3β-ol, stigmast-5-en-3β-ol, and stigmastadien-3β-ol).

DMDS/I_2 Derivatization of Extracts The DMDS derivatized *n*-alkenes present in waxes, cephalic, and abdomen extracts of males and workers of *P. droryana* and *N. testaceicornis* were analyzed by GC-MS. Mass spectra of the DMDS derivatized *n*-alkenes displayed fragment *m/z* 173 (94%, $[CH_3(CH_2)_7CH = SCH_3]^+$), indicating the double bond position at C-9, and base peaks (100%) at *m/z*: 243 $[CH_3(CH_2)_{12}CH = SCH_3]^{+\cdot}$ (*n*-C23); 271 $[CH_3(CH_2)_{14}CH = SCH_3]^{+\cdot}$ (*n*-C25); 285 $[CH_3(CH_2)_{15}CH = SCH_3]^{+\cdot}$ (*n*-C26); 299 $[CH_3(CH_2)_{16}CH = SCH_3]^{+\cdot}$ (*n*-C27); 313 $[CH_3(CH_2)_{17}CH = SCH_3]^{+\cdot}$ (*n*-C28); 327 $[CH_3(CH_2)_{18}CH = SCH_3]^{+\cdot}$ (*n*-C29); 355 $[CH_3(CH_2)_{20}CH = SCH_3]^{+\cdot}$ (*n*-C31); and 383 $[CH_3(CH_2)_{22}CH = SCH_3]^{+\cdot}$ (*n*-C33). The DMDS derivative of *n*-hentriacontadiene had two sets of fragments at *m/z* 173/353 $[CH_3(CH_2)_7CH = SCH_3]^{+\cdot}/[CH_3(CH_2)_8CH{=}CH(CH_2)_{10}CH = SCH_3]^{+\cdot}$ and *m/z* 187/339 $[CH_3(CH_2)_8CH = SCH_3]^{+\cdot}/ [CH_3(CH_2)_7CH = CH(CH_2)_{10}CH = SCH_3]^{+\cdot}$, indicating the presence of double bonds at C-9 and C-21.

Field Test Workers from three of seven stingless bee species, i.e., *P. droryana* (*N*=4), *N. testaceicornis* (*N*=2), and *Frieseomelitta silvestrii* (*N*=6), were attracted to paper strips containing the test compounds. The stingless bees flew around the strips of paper containing (*S*)-(+)-2-heptanol and the mixture of (*S*)-(+)-2-heptanol/(*S*)-(+)-2-nonanol (1:1). They did not fly around the paper containing the racemic mixture of 2-heptanol, 2-nonanol, and 2-undecanol, and rarely landed on these strips—if so, only for very short periods. Aggressiveness was not observed. Males were present in the nests and flying outside, but were not attracted.

Discussion

Nest construction and colony organization were performed by queens and workers upon detection of chemical signals. Stingless bee males have restricted tasks. Two to 3 week-old males stay inside the colony helping with building activities, trophallaxis, nectar dehydration, incubation, and nest defense. As adults, they leave the colony, but temporarily congregate nearby waiting for a queen to fly out from the nest (Roubik 1989; Velthuis et al. 2005). Male communication signals have not been investigated thoroughly, but this topic merits investigation.

The overall chemical composition of both cuticular waxes and cephalic extracts from *N. testaceicornis* males and workers are similar. However, minor compounds distinguish one sex from the other—e.g., phenyl tridecane is only present in male cuticular waxes (Table 1). Nonanal, pentacane, pentadecene, 9-tricosene, and 7,11,15-trimethyl-3-methylene-1,6,10,14-hexadecatetraene are present in the cephalic secretion of *N. testaceicornis* workers, distinguishing them from males that do not contain these constituents (Table 1).

Interspecies chemical comparisons revealed that the predominant constituents in *N. testaceicornis* cuticular waxes were heptacosane, (*Z*)-9-nonacosene, and 9,21-hentriacontadiene. In *P. doryana*, the predominant hydrocarbons were *n*-pentacosane and (*Z*)-9-heptacosene. These compounds help to differentiate chemically between these stingless bee species (Table 1). Insect cuticular hydrocarbons are important surface pheromones in inter- and intraspecific communications, mainly in nestmate recognition (Singer 1998; Abdalla et al. 2003). Thus, our results are consistent with the use of hydrocarbons in recognition systems.

Cuticular waxes of *P. droryana* workers and males were similar, but the cuticular waxes of workers had more terpenes and nerolidyl acetate than males (Table 1). Cuticular waxes from workers and males are similar in *P. droryana* and *N. testaceicornis*. This also was observed in workers and males of *Melipona scutellaris* (Kerr et al.

2004). Therefore, it seems to be common in stingless bees, where cuticular hydrocarbons of males and workers of the same species have similar chemical compositions.

The cuticular components of social insects are both individually produced (mainly by glands) or acquired from environmental sources (food, nest material, and other sources) (Moritz and Heisler 1992; Downs and Ratnieks 1999). Therefore, they generally are used as cues for nestmate recognition, and each colony has its own chemical template (Nunes et al. 2008). This explains why workers and males have many cuticular chemical similarities.

Abdomen extracts of *N. testaceicornis* workers consist of terpenyl acetates (approx. 84%), with geranylgeranyl acetate as the main constituent, while males secret linear *n*-alkanes, *n*-(*Z*)-9-alkenes, *n*-9,21-hentriacontadiene, and some sesquiterpenes (Table 1). Geranylgeranyl acetate was detected previously in extracts of the abdominal Dufour glands from *N. testaceicornis* workers (Cruz-López et al. 2001). This diterpene ester is absent in male extracts; the main chemical difference between *N. testaceicornis* abdomen extracts of males and workers. Abdomenal extracts of *P. droryana* males and workers were distinct. Large quantities of long chain aldehydes (tetradecanal and hexadecanal) and terpenic alcohols [(*E*,*E*)-farnesol, (*E*,*Z*)-farnesol, and geranyl linalool] were present in abdomen extracts of workers, while the major constituents in male abdomen extracts were (*Z*)-9-heptacosene and fatty acids (linoleic and linolenic acids, present in about 40% of the abdomen extracts) (Table 1). Therefore, workers and males of *P. droryana* and *N. testaceicornis* could be distinguished easily by the chemical composition of their abdomen extracts (Table 1).

The chemical differences between abdominal extracts of *N. testaceicornis* and *P. droryana* workers and males are remarkable as both have distinct constituents. *N. testaceicornis* has geranylgeranyl acetate and (*Z*)-9-nonacosene, respectively. Workers and males of *P. droryana* have tetradecanal and unsaturated fatty acids (linoleic and linolenic acids), respectively (Table 1). Abdominal extracts not only distinguish one species from the other, but also the sexes. The Dufour gland is not present in males, and is probably responsible for the striking chemical differences in abdomenal extracts (Abdalla et al. 2004). The Dufour gland also is absent in workers of *Trigona*, *Oxytrigona*, *Cephalotrigona*, *Scaptotrigona*, *Partamona*, and *Lestrimelitta* (Lello 1976; Patricio et al. 2003). However, all species of the *Plebeia* group, including *P. droryana*, have Dufour glands (queens and workers). *N. Testaceicornis* is distantly related to the *Plebeia* group, and Dufour glands are present in queens and workers. Dissection of the abdomens of *N. testaceicornis* and *Plebeia* sp. workers (later identified as *Plebeia droryana*) revealed a large sac at the end of the abdominal tip with a duct opening into the vagina (Patricio 1995; Cruz-López et al. 2001). The Dufour gland in *N. testaceicornis* is lined with a single layer of Type I secretory cells (Noirot and Quennedey 1974) with a peculiar cranelate epicuticle and vesicular smooth endoplasmic reticulum, as shown by transmission electron microscopy images (Patricio 1995). Preliminary studies of Dufour gland secretions from *Plebeia* sp. revealed tridecane, pentadecane, hexadecane, farnesol, and geranyl geraniol; however, not all constituents have been identified (Patricio 1995).

Both species also can be differentiated by cephalic extracts. *N. testaceicornis* male and worker extracts are characterized by 9,21-hentriacontadiene and long chain esters (hexadecyl hexadecenoate and hexadecyl octadecenoate), which are absent in *P. droryana* (Table 1). The short-chain esters in *N. testaceicornis* and *P. droryana* cephalic extracts of foraging workers also were detected in eleven Brazilian stingless bee species (Francke et al. 2000). However, most long chain esters (tetradecyl dodecanoate,

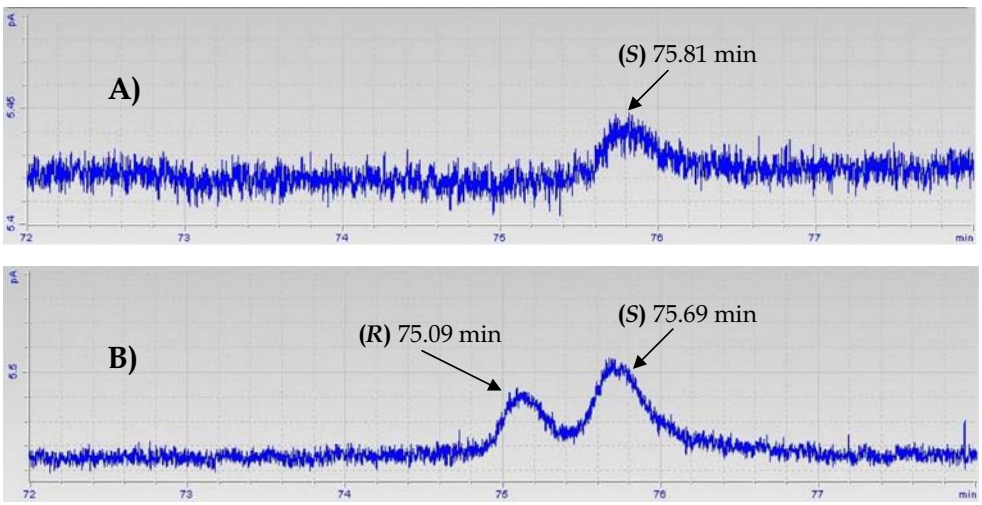

Fig. 1 Absolute configuration of natural 2-nonanol from cephalic extracts of *Plebeia droryana* males determined by GC-FID with chiral column (Chrompack CP-Chirasil-Dex CB; 25 m×0.25 mm×0.25 μm; 50°C to 100°C at 0.4°C/min). A) natural 2-nonanol from cephalic extracts of the *P. droryana* males; and B) 4:1 mixture of cephalic extracts of *P. droryana* males and synthetic (±)-2-nonanol

hexadecyl hexadecenoate, hexadecyl hexadecanoate, hexadecenyl octadecenoate, and hexadecyl octadecenoate) had not previously been detected in stingless bees (Table 1). These long chain esters can play specific roles in the chemical communication of stingless bees. This is similar to hexyl decanoate, the trail pheromone of *Trigona recursa* foraging workers (Jarau et al. 2006).

n-Alkanes and *n*-alkenes were more abundant in cephalic extracts of *P. droryana* workers, while steroids were more abundant in male cephalic extracts, thus differentiating the cephalic extracts of these two sexes. A spiroacetal, (*E,E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (Kitching et al. 1989; Booth et al. 2006), was found in male and worker cephalic extracts of *N. testaceicornis*. Other spiroacetals are known as alarm pheromones in rove beetles, fruit flies, and wasps (Fletcher and Kitching 1995; Zhang et al. 1999; Bruschini et al. 2006, 2008).

(*S*)-2-Nonanol is a component in the cephalic extracts of *P. droryana* males. Its absolute configuration was determined by chiral-gas chromatography (Fig. 1) using (±)-2-nonanol and (*S*)-2-nonanol to optimize the chromatographic conditions and confirm the (*S*) absolute configuration of the compound—a novel result. Curiously, the 2-alkanols were not detected in cephalic and abdominal extracts of *P. droryana* and *N. testaceicornis* workers. Thus, these are chemical signals used by males and/or queens and detected by workers. 2-Alkanols have also been detected in cephalic extracts of the workers from several stingless bee species (*Trigona hyalinata, T. spinipes, T. hypogea, T. truculenta, Tetragona clavipes, Frieseomelitta trichocerata, F. xanthopleura, F. silvestrii languida, Tetragonisca angustula, Geotrigona mombuca*, and *Lestrimelitt*a *limao*). This suggests that they might act as alarm pheromones (Luby et al. 1973; Johnson et al. 1985; Francke et al. 2000). However, in our experiments, only workers of *P. droryana*, *N. testaceicornis*, and *Friesiomelitta silvestrii* were attracted to (*S*)-(+)-2-heptanol and to the mixture of (*S*)-(+)-2-heptanol/(*S*)-(+)-2-nonanol (1:1), while a racemic mixture of secondary alcohols [(±)-2-heptanol, (±)-2-nonanol, and (±)-2-undecanol] was not attractive. Males did not respond to these alcohols. The selective attraction exerted by the chiral 2-alkanols suggests that the (*S*)-2-alkanols convey a specific signal between the sexes. Worker bees behaved as if they were searching for something while flying around the pieces of paper, but were not aggressive. This observation suggests that these are not alarm or recruting pheromones, but rather a chemical signal detected by workers. However, we did not detect this component in *Plebeia droriana* worker extracts only in male cephalic and abdomenal extracts, indicating that the worker bees detect, but do not emit, this chemical signal. Thus, the differentiation of the chemistry of workers and males of these two bee species was important to detect signals produced by one and both sexes.

The large differences in worker and male head and abdominal and extracts for both species were assigned to the presence of specific glands with different compositions according to sex, caste, or age (Cruz-Landim and Mota 1993; Gracioli-Vitti et al. 2004). The chemical signals from the head and abdomen may play important functions in the colonial context as caste and sex recognition.

In summary, we have shown for the first time the *N. testaceicornis* and *P. droryana* interspecific chemical differences and the chemical constituents that differentiate males from workers that might be useful in future behavioral studies of stingless bees'.

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germination and growth of wheat (*Triticum aestivum*) and *Phalaris minor* in laboratory bioassays. The essential oils of *L. stoechas* and *L. angustifolia* also have been determined to have phytotoxic effects on the seedling growth of *A. retroflexus* and *Portulaca oleracea* that infest tomato (*Lycopersicon esculentum*) and cotton (*Gossypium* spp.) crops (Argyropoulos et al. 2008). In addition, volatile compounds from *L. angustifolia* have a negative effect on the germination of common cocklebur (*Xanthium strumarium* L.), sterile wild oat (*Avena sterilis* L), and short spiked canarygrass (*Phalaris brachystachys* L.) (Uremis et al. 2009).

There are several reports on the chemical constituents of extracts and essential oils from various *Lavandula* spp. Chemical classes represented in the species include phenolics (Areias et al. 2000) and terpenes (Salido et al. 2004; Aburjai et al. 2005). Tiliacos et al. (2008) determined that a substantial component of *Lavandula intermedia* is made up of two compounds, coumarin and herniarin (7-methoxycoumarin). Angioni et al. (2006) determined via gas chromatography and mass spectrometry (GC/MS) that the main component of essential oils from *L. stoechas* is fenchone, a monoterpene that accounts for 52.6% of the leaf/stem and 66.2% in flowers. Another terpene, camphor, accounted for 13.1% and 27.1%, respectively.

It is not known, however, which compounds are involved in the phytotoxicity of *Lavandula* extracts. In addition, no published papers were found on the effect of *Lavandula* spp. on germination and seedling growth of ARG, other than our initial screening work presented in Haig et al. (2005). The current study aimed to determine the broader potential of the compounds found in lavender extracts in order to find new potential natural herbicides. Several lavender species and their individual plant parts were tested to determine the extract of highest potency. This extract was subjected to bioassay guided fractionation, and its chemical constituents analyzed to determine novel herbicide chemistries.

Methods and Materials

Aqueous Extract Preparation Several species of lavender (*Lavandula allardii*, *L. stoechas*, *L. x intermedia*, *L. dentata, L. angustifolia, L. x heterophylla*, and *L. viridis*) were collected near Charles Sturt University, Wagga Wagga, NSW and dried for 72 h at 40°C. Samples were divided into stem and leaf tissues and flower sub-samples prior to drying. Dehydrated plants and individual parts were milled into a fine powder through a 1 mm sieve. Ten grams of lavender powder were added to 100 ml of sterile, deionized water and placed in a dark incubator at 20°C for 3 d. Extracts were swirled gently by hand for 30 sec each day. The resulting extract was filtered through 4 layers of muslin cloth, vacuum-filtered through Whatman #1 filter paper, and sterilized via a 0.25 μm filter. This extract was considered to be the full strength extract (100%). Additional concentrations used in these experiments were obtained via serial dilution of the full strength extract.

Bioassays of Plant Extracts Commercial herbicide-susceptible *L. rigidum* (HS-ARG) seeds were sterilized for 2 min with 2.5% NaClO, rinsed thoroughly with distilled water, and placed in a 25°C incubator (12 h light/12 h dark) for 2 d. Fifteen pre-germinated ARG seeds were sown in petri plates lined with Whatman #1 filter paper that contained 4 ml of plant extract or distilled water in the case of the control. Seven extract concentrations were used: 0.1, 1, 5, 10, 25, 50, and 100% and a water control. After 3 d of incubation, seedling root and shoot lengths were measured. Three replications were arranged using a randomized complete block design. An identical experiment also was conducted using samples of ARG with known resistances to several modes of action that involve acetyl CoA carboxylase (ACCase) inhibitor resistance, ACCase inhibitors plus glyphosate resistance (HR-ARG), ACCase inhibitory plus acetolactate synthase (ALS) inhibitor herbicide resistances, and triazine resistance.

To determine whether lavender phytotoxicity was species specific, the most phytotoxic extract, *L. x intermedia* cv. Grosso, also was tested against canola (*Brassica napus* L.), wheat (*Triticum aestivum* L.), and subterranean clover (*Trifolium subterraneum* L.). Screening was similar to that described previously, with one exception. Wheat and subterranean clover were surface sterilized for 15 min prior to pre-germination rather than the 2 min for ARG and canola.

Stability of Extract Three 400 ml aliquots of 100% *L. x intermedia* cv. Grosso were stored in 500 ml glass Schott bottles under the following conditions: 4°C in the dark, 25°C in continuous light, and 25°C dark for a total of 256 days. To monitor phytotoxicity and determine the occurrence of basic chemical transformations within the 256 day period, pH and electrical conductivity were recorded on day 2, 4, 8, 16, 32, 64, 128, and 256 with a TPS digital pH meter and an Activon Pocket Conductivity Meter. On each sampling day, the original extract (100%) was serially diluted to concentrations of 50, 25, 10, 5, 1, 0.1, and 0% (control). The bioassay procedure was identical to that described above.

Solvent Extraction A large volume of *L. x. intermedia* cv. Grosso was prepared for fractionation and chemical

analysis by adding 175 g of dried powder to 1,750 ml of sterile, distilled water in a 2 L Schott bottle. The mixture was stored in the dark for 72 h at 20°C prior to filtering through 4 layers of muslin cloth, followed by vacuum filtering through one layer of 90 mm Advantec # 2 filter paper to remove fine particles. A 0.25 µm filter was used to sterilize the solution. The resulting 1,200 ml was sequentially partitioned with five solvents of increasing polarity: hexane, diethyl ether, dichloromethane, ethyl acetate, and *n*-butanol. Four aliquots of 300 ml of solvent were washed through the aqueous layer to exhaustively remove compounds of like polarity. The five solvent fractions were dried by the addition of anhydrous Na_2SO_4, and rotary evaporated to dryness at 40°C.

Chemoassay of Solvent Fractions Phytotoxicity bioassays were undertaken for each of the five solvent fractions, in addition to the aqueous residues, before and after solvent extraction using the bioassay procedure described. Concentrations of 20, 200, and 1,000 ppm were prepared by dissolving the dried solvent fraction in methanol. Four ml of each concentration were added to a 9 cm glass petri-plate that contained Advantec #2 filter paper. For the control, methanol only was added to the control dish and allowed to evaporate. After the methanol had completely evaporated from every plate, 4 ml of sterile, deionized water were added to each dish. A randomized complete block design was employed, with 3 replications, using 15 pre-germinated ARG seedlings per plate. Root and shoot growth were measured after incubation for 72 h in the dark at 25°C.

Column Liquid Chromatography (LC) Fractionation The most phytotoxic solvent fraction (hexane) underwent more focused phytotoxicity-guided isolation by using traditional column liquid chromatography (LC) to target the most herbicidal compounds, hence making the identification task that used spectroscopy more practical. The LC column was 200×37 mm i.d. and contained a packing of 200–400 mesh silica gel with a flow rate of 50 ml/8 min. Hexane fraction evaporate (0.37 g) was added to the top of the column for gradient elution. By using gradient elution, the polarity of the liquid mobile phase was altered gradually from non-polar (hexane) to increasingly more polar (methanol) (Table 1). Overall, 38×50 ml sub-fractions were collected from the initial hexane fraction. Vials containing enough mass for bioassays were made up in methanol to concentrations of 20, 200, and 1,000 ppm.

GC/MS Conditions A Varian 3400 CX gas chromatograph (GC) was coupled with a Varian Saturn 2000 ion trap mass spectrometer (MS). The GC was fitted with a DB-5 (30 m× 0.32 mm i.d.×1 µm) fused silica capillary column (J&W Scientific). The programmed column temperature was set at 40°C and held for 1 min, then increased to 70°C at 10°C/min for 3 min, increased to 125°C at 5°C/min for 11 min, then increased to 240°C at 10°C/min for 11.5 min, held at 240°C for 10 min, increased to 290°C at 50°C/min for 1 min, held at that temperature for 5 min. Ultra-pure helium was used as carrier gas (linear velocity of 34 cm/s). One µL sample splitless injections were undertaken at the injection temperature of 280°C. The Varian Saturn 2000 MS detector employed the electron ionization mode. The MS was programmed to scan a mass range of 50 to 650 m/z every 0.700 sec with a solvent delay time of 4.5 min. Data were analyzed by using the Varian (Walnut Creek, CA, USA) Saturn Chromatography Work Station software (Version 1.3), and tentative identification of chemicals in the solvent fractions were made from the NIST 2005 mass spectral library database. Pure reference standards were injected to confirm identification of specific chemicals having a high fit of above 900 (out of a possible 1,000) in the NIST database. A high match with the reference standard mass spectrum and an identical retention time indicates the presence of these chemicals.

Table 1 Mobile phase gradient elution for LC

Vial #	Gradient
1–10	100% hexane
11–14	1% ethyl acetate in hexane
15–18	3% ethyl acetate in hexane
19–22	5% ethyl acetate in hexane
23–26	10% ethyl acetate in hexane
27–30	20% ethyl acetate in hexane
31–32	50% ethyl acetate in hexane
33–34	100% ethyl acetate
35–36	100% methanol
37–38	100% methanol

Infrared Analysis To confirm MS identification of phytotoxic compounds, IR spectral fingerprinting was undertaken. The Pye-Unicam SP3-100 Infrared Spectrometer had a 0.1 mm path length, and the scan was done from the 4,000 to 700 cm^{-1} range. Less than 0.2 g of sample were dissolved in analytical grade chloroform.

Chemoassay of Coumarin and its Structural Derivatives Coumarin was compared against 18 of its structural derivatives across the concentration range: 1, 5, 10, 25, 50, and 100 ppm. Pure reference standards of 18 coumarin structural derivatives were purchased from Sigma-Aldrich, Australia and dissolved in AR methanol to obtain concentrations of 1, 5, 10, 25, 50, and 100 ppm by using serial dilution. Four ml of each coumarin-derivative concentration were added to glass petri-dishes, lined with Advantec #2

filter paper. After the methanol had evaporated, 4 ml of sterile, deionized water were added to each dish. The remainder of the bioassay was conducted as described above.

Soil Trials In order to minimize probable volatilization of constituents before effects on soil or plant, incubator conditions were lowered to 15°C, with 12 h L: 12 h D.

Lavender Extract To assess the effect of *L. x intermedia* cv. Grosso extracts in soil where myriad microbial interactions and soil processes are occurring in contrast to agar based bioassays, bioassays were conducted in plastic 4 cm diam, 10 cm long cylindrical vials (50% soil:50% peat moss). Ten pre-germinated ARG seeds were sown evenly in the vials, which had been pre-moistened with 10 ml tap water and covered with 0.2 cm soil. Nine days after sowing, ARG seedlings were sprayed with 3.5 ml of either control, 10, 25, 50, or 100% extract with a hand-held aspirator. Every 3 d over 3 wk, 3.5 ml of water were added to each vial to prevent wilting. Total germination counts were tabulated and above-ground ARG seedlings were harvested prior to drying for 72 h in a 70°C plant dehydrator. Dry weights were measured for statistical analyses. Three replicates were arranged in a complete randomized block design.

Coumarin Coumarin concentrations used were 0, 125, 250, 500, and 1,000 ppm. Each concentration, including the control, contained a 5% methanol −95% deionized water ratio. Methanol initially was used to dissolve semi-water soluble coumarin before water was added. The 5% MeOH −95% DI water mixtures were sprayed onto the soil as described for the lavender extract pot trial. All concentrations were done in triplicate, in a randomized complete block design.

Statistics The majority of the ARG root and shoot length data from bioassays and chemoassays was analyzed via ANOVA using Genstat® v.11. Data presented in Fig. 1, root and shoot measurements, were converted to % of control values prior to analysis to facilitate a common starting point for the different species for ease of comparison. Least significant differences (LSD) at $P=0.05$ for all data were calculated to enable comparisons. For the pot trial experiments, dry weights also were subjected to analysis of variance.

Results

Bioassays Since root growth appeared to be a more sensitive parameter than shoot growth, only root growth data are presented. Not all lavender species had the same phytotoxic potential. Overall, *L. x intermedia* cv. Grosso was the most phytotoxic species ($LSD_{species}=1.8$, $P<0.001$). Table 2 demonstrates that the stem and leaf extract of *L. x intermedia* cv. Grosso was the most phytotoxic against ARG seedlings ($LSD_{species*extract}=2.6$, $P<0.01$). This extract inhibited ARG root growth by >94% compared with the control. All extracts of all species except for *L. stoechas* and *L. viridis* inhibited ARG root growth by >70%. As the stem and leaf extract of *L. x intermedia* cv. Grosso was the most phytotoxic, the remainder of the research was conducted with this extract.

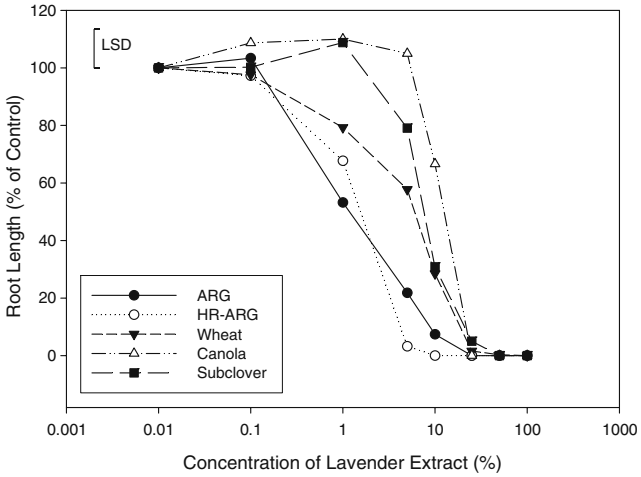

Fig. 1 Effect of *Lavandula x intermedia* cv. Grosso on root growth of several plant species ($LSD_{interactions}=13.4$, $P<0.001$)

When this extract was tested on additional plant species, it was phytotoxic to multiple species (Fig. 1). All resistance samples were equally susceptible, and only results for the ACCase plus glyphosate resistant sample (HR-ARG) are included. Overall, lavender was increasingly phytotoxic towards test species as follows: canola < subterranean clover < wheat < HS-ARG = HR-ARG. There were differences in root growth between each level ($LSD_{species}=4.5$, $P<0.001$). Root growth of HS-ARG, was not significantly different from HR-ARG, however, overall they were most inhibited by *L. x intermedia* cv. Grosso extract. At concentrations of 25% extract and above, there were no significant differences in root growth among species. Maximum differences in suppression of root growth were seen at 5 and 10% extract concentrations ($LSD_{spp\ x\ conc}=13.4$, $P<0.001$). There were no significant differences among species at concentrations less than 1%.

Stability of Extracts Figure 2 shows the effect of the 1% cv. Grosso extract on ARG root growth over time. Although there was a significant change in phytotoxicity over time under different storage regimes ($LSD_{interactions}=7.3$, $P<$

Table 2 Effect of 10% extracts from different *Lavandula* species on annual ryegrass root growth

Lavandula species	ARG root length (mm)[a]	
	Stem & leaf extract	Flower extract
L. x intermedia cv. Grosso	3.1	9.3
L. x heterophylla	5.8	13.9
L. angustifolia cv. Edgerton Blue	7.7	15.3
L. x allardii cv. Mitchum	8.6	16.1
L. dentata cv. Candicans	13.4	19.0
L. viridis	39.1	40.3
L. stoechas cv. Fairy wings	49.8	52.8

LSD $_{spp * extract}$=2.6, P<0.01

[a] distilled water control mean=58.9 mm

0.001), by day 128, all three extracts had similar phytotoxicities, regardless of storage conditions. All extracts reduced ARG root growth by 40%. In no treatment was the phytotoxicity lost between day 2 and day 256. While phytotoxicities of both the 25°C light and 4°C dark treatments did not change significantly, the 25°C dark treatment increased significantly when comparing day 2 and day 256. Average root length in this treatment decreased from 41 to 26 mm.

The pH of the 100% extracts stored under different conditions varied from 5.0 to 6.0 over 256 days, while the electrical conductivity (EC) of the 100% extracts ranged from 5.3–6.3 dS/m. The observed effect on root growth is more likely due to presence of extract rather than pH or EC effects, as the latter two are well within reported limits suitable for plant growth.

Chemoassay of Solvent Fractions There were differences in the phytotoxicity of the solvent fractions (LSD=2.7, P< 0.001). From least to most toxic, the extracts ranked as follows, with phytotoxicity increasing significantly between each extract: remaining extract, dichloromethane, ethyl acetate, *n*-butanol, diethyl ether, and hexane. The effect of concentration also was significant (LSD=1.9, P<0.001). Figure 3 shows the response curve for each fraction (LSD $_{interactions}$=5.3, P<0.001). The hexane fraction inhibited ARG root growth by 61% at 20 ppm, whereas the diethyl ether extract inhibited it by 16% at 20 ppm and ~99% at 200 ppm. The ethyl acetate and *n*-butanol extracts inhibited ARG root growth by <10%, while the dichloromethane extract actually stimulated ARG growth at this concentration (20 ppm).

Chromatography When the hexane fraction was further fractionated, most of the eluent mass was found in sub-fractions 33 and 34. A green crystalline solid was observed in sub-fraction 33, while there was a white crystalline solid in sub-fraction 34. Comparisons with pure reference standards and near-infra red analysis confirmed that sub-fraction 33 consisted entirely of coumarin while sub-fraction 34 consisted entirely of 7-methoxycoumarin. The dose response curves are plotted on Fig. 3. Sub-fraction #33 inhibited root growth by 89% at 20 ppm, whereas sub-fraction #34 was much weaker, with only 25% inhibition at

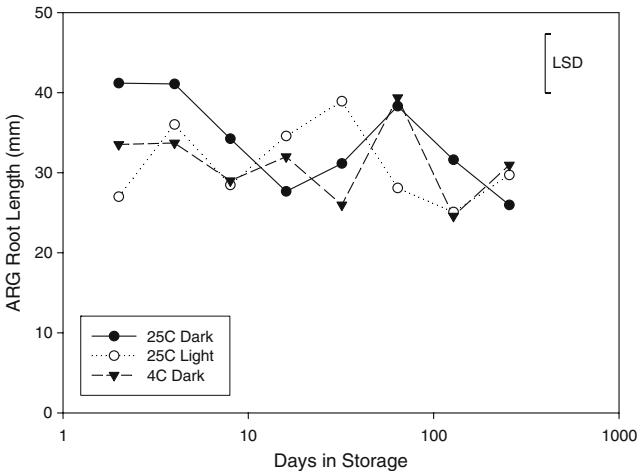

Fig. 2 Changes in phytotoxicity of *Lavandula x intermedia* cv. Grosso extract (1%) under different storage regimes (LSD $_{interactions}$=7.3, P<0.001)

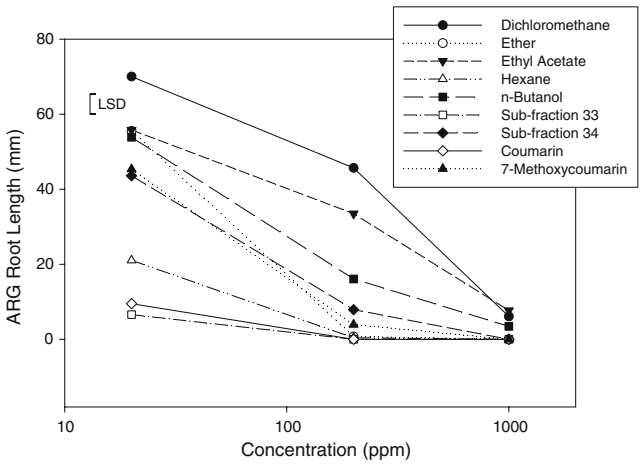

Fig. 3 Inhibition of annual ryegrass (ARG) root growth due to different solvent fractions and sub-fractions (LSD $_{interactions}$=5.3, P<0.001)

this concentration. A coumarin concentration as low as 20 ppm inhibited ARG root length by 84%.

Chemoassay of Coumarin and Structural Derivatives Dose-response data of the 18 pure reference samples tested are shown in Table 3. Coumarin demonstrated superior phytotoxic potential against ARG root growth ($LSD_{interactions}$= 3.6, $P<0.001$) although 7-hydroxycoumarin had a similar dose-response curve, providing 100% root inhibition at 100 ppm. Only 17 chemicals inhibited ARG root growth by >70% at the highest concentration used in this experiment, 100 ppm. Four chemicals inhibited ARG root growth by <20%.

Soil Trial Figure 4 shows the effect of *L. x intermedia* cv. Grosso extract on ARG shoot weight when sprayed post-emergence. All concentrations reduced ARG shoot weight compared with the distilled water control (LSD=0.45, $P<$ 0.001). Concentrations above 10% reduced ARG shoot weight by more than 50%. The 100% extract reduced shoot weight by almost 90%.

The coumarin dose-response curve in soil is shown in Fig. 5. All concentrations of coumarin reduced ARG shoot weight compared with the control (LSD=0.40, $P<0.001$). Concentrations of 250 ppm and above inhibited ARG shoot weight by <50%. A concentration of 1,000 ppm reduced ARG shoot weight by almost 80%.

Discussion

In this study, multiple *Lavandula* species were tested for phytotoxic effects on ARG growth in an attempt to discover potential natural herbicide leads. *Lavandula stoechas* was the least phytotoxic species, inhibiting ARG root growth by <20%. The stem and leaf extract of *L. x intermedia* cv. Grosso, inhibited ARG growth by 94%. This high phytotoxicity was observed for multiple species. When extracts were tested for stability, there was no loss of phytotoxicity over a 256 day period. This is an important consideration for potential future users who generally would keep herbicides between cropping seasons prior to application.

Although the plant extract itself had been known to have potential as a herbicidal treatment, one aim of this research was to isolate specific phytotoxic compounds. Coumarin was the main constituent of the most herbicidal sub-fraction of the highly toxic hexane fraction, and was active at low concentrations. Coumarin is a well known phytotoxin that has been tested in laboratory studies for its effect on germination and growth of wild oats (*Avena fatua*) (Goodwin and Taves 1950), timothy grass (*Phleum pratense*) (Avers and Goodwin 1956), beggar's ticks (*Bidens pilosa*) (Pergo et al. 2008), barnyard grass (*Echinochloa crus-galli*) (Chon and Kim 2004), alfalfa (*Medicago sativa*), velvetleaf (*Abutilon theophrasti*), and Italian ryegrass (*Lolium multiflorum)*

Table 3 Effect of coumarin structural derivatives on annual ryegrass (ARG) root growth

Chemical	ARG Root Length (mm)[a]					
	1ppm	5ppm	10ppm	25ppm	50ppm	100ppm
Coumarin	42.3	23.1	5.1	0.5	0.0	0.0
7-Hydroxycoumarin	49.4	32.1	18.7	0.5	0.0	0.0
7-Diethylamino-4-methylcoumarin	41.7	33.8	30.1	22.5	10.6	2.6
3-Hydroxycoumarin	45.2	42.9	31.8	21.8	16.2	11.7
4-Methylesculetin	37.2	33.1	34.1	29.8	19.9	11.8
7-Methoxycoumarin	48.3	41.8	39.7	36.4	29.4	11.9
Coumarin-3-carboxylic acid	50.8	46.8	42.4	36.7	28.5	20.6
3-Acetylcoumarin	52.1	48.4	42.5	43.0	41.3	36.0
7-Amino-4-methylcoumarin	48.1	48.8	43.8	44.4	37.4	31.1
8-Acetyl-7-hydroxycoumarin	49.8	44.3	44.6	35.2	25.0	12.0
Daphnetin	55.5	56.2	47.6	50.6	49.5	17.4
7,8-Dihydroxy-4-methylcoumarin	52.0	49.8	49.0	46.0	39.2	25.2
3-Aminocoumarin	55.7	47.9	49.4	45.1	41.0	22.3
7-Amino-4-(trifluoromethyl)coumarin	48.3	47.2	49.5	44.2	41.9	41.4
4-Hydroxycoumarin	51.4	47.8	52.4	47.2	37.2	34.9
5,7-Dihydroxy-4-methylcoumarin	54.9	56.1	53.1	54.4	48.0	52.4
Scopeletin	54.0	52.5	55.3	45.0	45.5	28.9
7-Hydroxycoumarinyl-4-acetic acid	59.0	58.5	58.8	54.7	55.0	51.2
7-Mercapto-4-methylcoumarin	61.4	62.7	60.9	51.7	54.2	67.1

$LSD_{chemical*concentration}$=3.6, $P<0.001$

[a] distilled water control mean = 53.0 mm

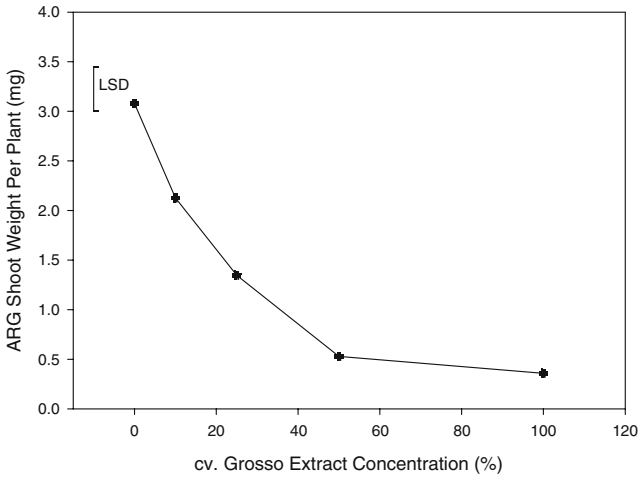

Fig. 4 Effect of *Lavandula x intermedia* cv. Grosso extract on annual ryegrass (ARG) when applied to soil post-emergence (LSD=0.45, $P<0.001$)

(Dornbos and Spencer 1990). Although coumarin has been tested on *L. multiflorum*, there are no literature reports of herbicidal properties against ARG, the most important weed in southern Australian winter wheat crops.

Due to coumarin's demonstrated potency against ARG, 18 structural analogues of coumarin were investigated to determine whether any variants of the coumarin lead structure, that consists of a unique coumarin heterocyclic ring system, enhanced ARG inhibition compared with coumarin itself. Coumarin remained the most biologically active compound tested. Low coumarin concentration of 20 ppm reduced ARG root growth by 84%.

The finding that coumarin was active on ARG with resistances to a range of herbicide modes of action suggests that its mode of action was different from herbicides to which resistance has occurred. Such a chemical option is desirable since evolution of herbicide resistance in ARG has threatened the viability of some existing options (Broster and Pratley 2006).

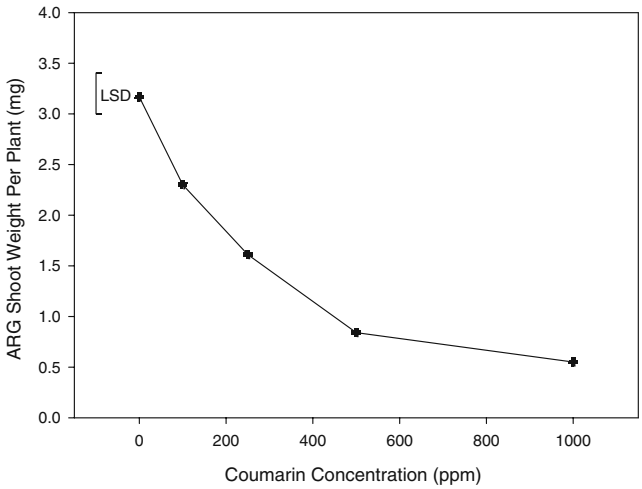

Fig. 5 Effect of coumarin on annual ryegrass (ARG) when applied to soil post-emergence (LSD=0.40, $P<0.001$)

Although lavender extract and pure coumarin bioassays both revealed promising herbicidal activities in the laboratory, the real test for potential herbicides is their effectiveness in the field where myriad soil characteristics and microbial interactions come into play. Dias et al. (1995) determined that aqueous extracts of *L. stoechas* inhibited germination of *Phalaris minor*, a weed of wheat crops, in laboratory bioassays. However, when Dias et al. (2004) conducted a soil pot trial with this extract, it was unsuccessful as a pre-emergence treatment. The authors suggested that factors such as light, microbial interactions, and metabolism were responsible for the loss in bioactivity in soil. Considering that many of the compounds previously identified in the literature are volatiles, it is likely that many extract components volatilize and are not available to exert an effect, whereas in petri plates, the volatiles are somewhat contained for a greater length of time and thus elicit an effect.

In this study, the lesser effect of extract in soil than that in the petri plate bioassay was expected due to myriad soil influences. However, both the lavender extract and coumarin had significant inhibitory effects on the growth of ARG in soil trials at all concentrations tested. The dose response curves (Figs. 4 and 5) are similar for both spray applications. This is not surprising as the most phytotoxic lavender fraction contained coumarin as the main active constituent.

Other natural herbicides have been found in plants. Leptospermone is a naturally occurring triketone with herbicidal properties in the Australian native, *Callistemon citrinus* (Cornes 2005). This compound was modified by Syngenta to make a potent synthetic derivative, mesotrione, which is almost 100 times more potent than the initial lead chemical (Cornes 2005). This herbicide (Callisto®) has been developed to control broadleaf weeds in (*Zea mays*) crops and is being used successfully as a pre-emergence and post-emergence herbicide (Cornes 2005). Thus, this approach offers promise in the search for alternative control options for weeds that are developing resistance to existing commercial herbicides, and could play a role in integrated weed management regimes.

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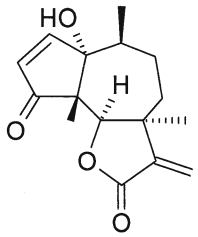
Table 1 Chemical properties of parthenin

formula	$C_{15}H_{18}O_4$
molecular weight	262.12 g/mol
chemical class	sesquiterpene lactone, pseudoguaianolide
nomenclature	1,6β-dihydroxy-4-oxo-10αH-ambrosa-2,11(13)-dien-12-oic acid-γ-lactone



has been identified as the most abundant inhibitor in an Indian population of *P. hysterophorus* (Kanchan and Jayachandra 1980), and its phytotoxicity has been verified on a variety of plant species (Datta and Saxena 2001; Belz et al. 2007). Although parthenin has been found in virtually all *P. hysterophorus* plant parts, it occurs most abundantly in trichomes on the surfaces of the leaves (Rodriguez et al. 1975; Reinhardt et al. 2004). By investigating the dynamics of parthenin accumulation in leaves of *P. hysterophorus*, Reinhardt et al. (2006) showed that the parthenin content of leaves increased with plant age, reaching a peak at the beginning of flowering. Accumulation of high levels of parthenin in or on leaves right until the end of the plant's life cycle suggested that a parthenin-mediated allelopathic potential in *P. hysterophorus* mainly relies on leaching of the compound from leaf residues, thus inhibiting or impeding the recruitment of other species, rather than leaching from leaf surfaces of actively growing plants. Simulating the natural release of inhibitors from decomposing leaf residues by aqueous extraction confirmed this assumption, as leaching of parthenin from leaf material high in parthenin was sufficient to account for up to 100% of the observed extract toxicity (Belz et al. 2007). Thus, if the success of *P. hysterophorus* in invading and establishing new territories actually involves residue allelopathy, parthenin should account for a major part.

Although the natural release of parthenin from leaf trash by way of leaching is not well characterized, the amount of parthenin that leaf material of a single senescent *P. hysterophorus* plant can introduce into the environment has been estimated at approximately 270 mg (Reinhardt et al. 2006). Thus, large quantities of parthenin could enter the soil through decomposing plant material, and estimated concentrations according to plant leaf contents ascribe an allelopathic potential in laboratory bioassays (Belz et al. 2007). However, knowledge of the amount of toxin released to the environment is merely a first step in proving a hypothesis of allelopathy (Fuerst and Putnam 1983). Once an allelochemical enters the soil environment, a number of interacting processes may take place, some of which may transform or degrade the compound and, thus, alter its activity. These processes are influenced by the nature of the compound, organisms present, soil properties, and other environmental factors (Cheng 1992). Therefore, proving a hypothesis of allelopathy must go beyond the production of phytotoxic concentrations by ensuring that the compound accumulates or persists long enough at levels sufficient to affect the development of other plants (Radosevich and Holt 1984). Although it is well-known that the behavior of allelochemicals in soils is a major determining factor for allelopathy, studies that investigate the accumulation of parthenin as well as its fate and persistence in soils are absent. The fact that soils previously infested with *P. hysterophorus* showed a plant growth inhibiting activity (Kohli and Batish 1994), suggests a possibility for direct effects of allelochemicals or their metabolites and/or the accumulation of phytotoxic levels over time. The prospect that parthenin is the major allelochemical responsible for this soil activity still necessitates understanding its soil behavior.

Therefore, the aim of this study was to investigate whether or not parthenin is sufficiently persistent, phytotoxic, and bioavailable in soils to cause an allelopathic effect that could contribute to the invasiveness of *P. hysterophorus*. We investigated these aspects by using soil samples from naturally invaded sites within the KNP, South Africa, that show long lasting *P. hysterophorus* infestation, as well as soil samples from adjacent, uninfested sites. Furthermore, we used commercially available soil samples employed, among other things, for degradation studies with pesticides. The role of various abiotic and biotic factors in the degradation of the compound was investigated, and phytotoxic levels required to directly affect the growth of target plants in a soil environment were quantified. The accumulation and bioavailability of parthenin in soil over time was further studied. This allows for a better understanding of the importance of parthenin as an allelochemical and ultimately for assessing the importance of allelopathy in the invasiveness of *P. hysterophorus*.

Methods and Materials

Origin of Parthenin Parthenin was extracted from *P. hysterophorus* plants (seeds collected at Skukuza in the KNP, South Africa) that were germinated under greenhouse conditions (15/9 h L:D, 22/18°C, 300/0 μE/m^{2}s) in a one-to-one mixture (*v*/*v*) of quartz sand and garden soil (Humusoil, Floragard, Germany) at the University of Hohenheim, Germany. Parthenin was isolated from organic extracts (*tert*-butyl methyl ether) of the leaf surface and fractionated by preparative high performance liquid chromatography (HPLC; Varian model chromatograph) with UV detection (Varian UV-VIS detector model 345) as described by Belz et al. (2007). Purity of isolates was quantified by HPLC-DAD (photodiode array detection) at ≥95%.

Origin of Soils Top soil samples (0–20 cm) were collected from various sites and included an organic soil (compost soil; CS), three 'standard soils' with certain characteristics provided by LUFA Speyer (Landwirtschaftliche Untersuchungs- und Forschungsanstalt Speyer, Germany), and four soil batches from the KNP, South Africa (Table 2). Samples were sieved at 2.0 mm and either used field-fresh (CS) or after storage at room temperature at 20% water-holding capacity (WHC). All stored soil samples were preincubated for 2 wk under test conditions (40% WHC, 20±2°C, darkness) prior to the start

Table 2 Physico-chemical properties of experimental soils

soil	sampling site	soil type[a]	clay silt sand[b] [%]	C_{org} [%]	pH[c]	WHC[d] [g/100g]	CEC_{pot}[e] [mval/100g]	Storage[f] [days]
CS	Hohenheim University Germany *uncultivated*	high loamy sand	15.4 39.0 45.6	5.12	6.9	54.7	23	0
2.1[g]	Dudenhofen LUFA Speyer[h] Germany *uncultivated*	poor silty sand	3.7 10.0 86.1	1.21	6.1	34.7	7	12
3A[g]	Altluβheim LUFA Speyer Germany *meadow*	poor sandy loam	17.6 41.1 41.3	2.20	7.1	49.4	19	12
5M[g]	Mechtersheim LUFA Speyer Germany *uncultivated*	medium loamy sand	11.5 34.7 53.8	1.56	7.1	42.1	13	12
SA1	Skukuza KNP[i] South Africa *uncultivated PTNHY[j] infested*	high sandy loam	19.2 19.6 61.2	3.68	7.16	41.9	30	79
SA2	Skukuza KNP South Africa *uncultivated*	pure sand	1.7 8.8 89.5	2.03	7.46	36.8	8.1	79
SA3	Crocodile Bridge KNP South Africa *uncultivated*	medium loamy sand	10.1 21.6 68.3	0.96	5.8	37.0	4	79
SA4	Crocodile Bridge KNP, South Africa *uncultivated PTNHY infested*	poor clay loam	28.3 31.6 40.1	1.03	6.7	47.8	22	79

[a] According to German DIN

[b] particle size (mm): <0.002 clay, 0.002–0.063 silt, 0.063–2.0 sand

[c] 0.01 M $CaCl_2$

[d] gravimetric water holding capacity

[e] potential cation exchange capacity

[f] room temperature; 20% WHC

[g] LUFA standard soil

[h] Landwirtschaftliche Untersuchungs- und Forschungsanstalt Speyer

[i] Kruger National Park

[j] PTNHY *P. hysterophorus*

of the experiment. The physico-chemical properties of the soils as given in Table 2 were determined by the soil laboratory LUFA Speyer.

Soil Persistence Studies—General Protocol An equivalent of 50 g of dry soil was added to glass jars (290 ml Sturzglas with glass lids, Weck, Germany) and adjusted to 40% WHC with demineralized water. Parthenin dissolved in a one-to-one mixture of acetone and demineralized water (*v*/*v*) was added to each soil sample to obtain a parthenin concentration of 10 µg/g dry soil (10 µl acetone/g dry soil). This concentration seemed relevant to mirror a natural situation of invasion based on the calculated potential parthenin levels in top soil (≤2 cm) at a plant density of 1–4 plants/m^2 [9.6–43.0 µg/g dry soil; after Van der Laan (2006)] and the parthenin levels found by us in naturally infested soil samples (0.1–6.4 µg/g dry soil). After addition of parthenin, soil samples were stirred thoroughly with a spatula to ensure an even distribution. Loose-fitting glass lids that allowed air circulation were placed on each jar, and jars were kept at 20± 2°C and darkness in growth chambers. Demineralized water was replenished every 3–4 d to maintain samples at 40% WHC by differential weighing. Initial sampling was done immediately after application and after 1 h to determine recovery rates, and thereafter another 14 times at intervals adapted to record degradation under the various specific experimental modifications. One jar was sampled at each time and stored at −20ºC until analyzed. In order to determine the influence of abiotic and biotic factors, the general protocol was modified as follows.

Influence of Abiotic Factors The dependence on soil type was studied with three standard soils (2.1, 3A, and 5M; Table 2). Concentration dependence was evaluated with six initial parthenin concentrations ranging from 8 to 820 µg/g dry soil. The influence of soil moisture was assessed at 20, 40, and 80% WHC, and the influence of ambient temperature was studied at 20, 25, and 30°C.

Influence of Biotic Factors The significance of microorganisms was investigated through the sterilization and preconditioning of soils. Sterilization of CS soil was done by autoclaving for 60 min at 120°C (1× sterilized) and repeated after 2 wk of incubation under test conditions (2× sterilized). Sterile, demineralized water was used to adjust and replenish soil moisture, and all preparation steps as well as replenishment of water were done under sterile conditions. Incubation of soil samples during the test was, however, non-sterile. An artificial preconditioning treatment of CS soil was done under test conditions with parthenin at four different concentrations ranging from 0 to 1,000 µg/g dry soil. The time intervals for preconditioning were adapted to allow for a degradation of parthenin lower than the limit of detection (2 wk at 10 µg/g, 3 wk at 100 µg/g, and 5 wk at 1,000 µg/g). The hypothesis of a natural preconditioning for parthenin degradation in *P. hysterophorus* infested soils was investigated by using soil samples collected from naturally infested sites and adjacent, uninfested sites in KNP, South Africa (Skukuza, Crocodile Bridge).

Chemical Analysis Parthenin was analyzed by organic solvent extraction of soil samples. At first, demineralized water was added to obtain a final volume of 15 ml/50 g dry soil. No water was added to soil samples adjusted to 80% WHC. Subsequently, 85 ml of a one-to-one mixture of acetone and *tert*-butyl methyl ether (*v*/*v*) were given to each soil sample followed by 30 min shaking extraction on a mechanical shaker at 200 motions/min (KS501digital, Fa. IKA Labortechnik). A 30 min sedimentation period followed. The supernatant was filtered through approximately 40 g of a one-to-one (*v*/*v*) mixture of Na_2SO_4 and quartz (cryst., 0.6–1.2 mm, Fa. Roth). An aliquot of 40 ml was concentrated in a rotary vacuum evaporator at 40°C followed by vacuum centrifugation at 40ºC (RVC 2–25, Fa. Christ, with cooling trap) until a volume of 600 µl was obtained. After the addition of 400 µl acetonitrile (ACN), samples were centrifuged (20 min, 14,000 rpm), and transferred to HPLC vials. HPLC analysis was done on a Waters model chromatograph with DAD detection (photodiode array detector, Waters 991). A Synergi polar C-18 reversed phase column [250 mm by 4.6 mm (4 µm), Phenomenex, Germany] was used, and eluted with a gradient of 15% ACN and 85% Na_2HPO_4-buffer (1 mM, pH 2.4, 10% ACN) for 0–5 min (0.65 ml/min flow rate), 56% ACN and 44% Na_2HPO_4-buffer for 5–19 min (0.75 ml/min flow rate), 100% ACN for 19–27 min (0.75 ml/min flow rate), then reequilibrated to starting conditions. Injection volume was 50 µl.

Parthenin was identified and quantified at 220 nm by using external calibration curves. The retention time was 17.3±0.2 min. Instrumental limits of quantification were determined at 220 nm according to Frehse and Thier (1991) at a fortification range of 0.25–10 µg/ml based on standard solutions of parthenin. The limit of quantification was 2.18 µg/ml or 0.11 µg/g soil based on a 50 g dry soil sample. Recovery rates for the analytical procedure were 99.3±5.4% at a fortification level of 10 µg parthenin/g dry soil on average for all experimental soils.

Soil Phytotoxicity Studies Phytotoxicity of parthenin in different soils was studied in a germination assay conducted in glass petri dishes (4.5 cm diam.) with *Lactuca sativa* L. var. *capitata* cv. Maikönig as test species. The fast growing, evenly germinating receiver *L. sativa* was preferred as it was shown to be as comparable in sensitivity to parthenin

as to some indigenous species (Belz et al. 2007). The quantity of soil per petri dish was calculated to give 120% WHC with the applied 2 ml of test solution per dish (4.0 g dry soil/petri dish for SA1, 4.5 g for SA2, 4.6 g for SA3, and 3.4 g for SA4). The aqueous test solution contained parthenin in nine different concentrations to give 0–1.6 mg/g dry soil and 1% acetone as solvent (5 µl/g dry soil). Controls received water and acetone only. For each treatment, 15 seeds were placed on the soil surface, and petri dishes were sealed with parafilm. Each treatment was replicated three times, and petri dishes were positioned in a randomized design in a growth chamber (12/12 h, 24/18°C, 50/0 µE/m^{2}s). After 7 d, total seedling length was measured (≥1 mm), and concentration-response curves were calculated using Equation 1.

Soil Phytotoxicity over Time The biological activity of parthenin in CS soil over time was studied at an initial concentration of 700 µg/g dry soil. Nineteen soil samples were treated with parthenin and incubated following the above described general protocol for soil persistence. Treated soil samples were harvested continuously over a period of 3 wk (one jar at a time) and stored at −20ºC until used. Two untreated soil samples (controls) were incubated in the same way and harvested at the beginning and the end of the incubation period. After thawing, soil samples were kept under a hood for 2 h and stirred every 15 min in order to evaporate acetone residues. Subsequently, each soil sample was readjusted to 40% WHC and split. One part (25 g) was chemically processed for quantification of parthenin, and the remaining part was used for bioassays.

To bioassay the remaining toxicity of parthenin in the harvested soil samples, 3.5 g of soil (40% WHC) were weighed per petri dish in triplicate for each harvest time, and demineralized water was added to give 120% WHC (1.3 ml/petri dish). After the addition of 15 seeds/petri dish, dishes were sealed with parafilm and incubated as described above. Total seedling length was measured after 7 d (≥1 mm), and responses over time were modeled by using Equation 1.

Soil Accumulation Studies The possibility of an *accumulation* of parthenin in soil over time due to a periodic exposure was evaluated by a single, double, and triple application of 400 µg/g dry soil at a time interval of 38 d each. The experimental procedure followed the above described general protocol for soil persistence studies with CS soil.

Data Analysis Mathematical modeling of concentration-response curves in phytotoxicity studies as well as degradation curves of parthenin in soil was done by nonlinear regression analysis using the logistic Equation of Streibig (1988) (Equation 1).

$$y = c + \frac{d - c}{1 + e^{b*LN(x/\alpha)}} \quad (1)$$

- y expected response in bioassay or soil toxin concentration
- x toxin concentration in bioassay or incubation time in soil
- d expected response of untreated controls in bioassay or soil toxin concentration at time $x = 0$
- c expected response at indefinitely large concentrations in bioassay or soil toxin concentration at $x \rightarrow \infty$
- b rate of change around α
- α EC_{50} (effective concentration giving 50% response in bioassay) or DT_{50} (time where 50% of the applied toxin concentration disappeared)

In case of hormetic responses in bioassay, modeling used the extended logistic regression model of Cedergreen et al. (2005), whereas the rate of increase in responses at subinhibitory concentrations (a) was fixed to −0.5 according to the smallest residual sum of squares. Significance of hormesis was assessed by the 95% confidence interval for the estimate of the theoretical upper bound of the hormetic effect f and was given for $f>0$. Characteristic features of the hormetic response were derived by estimation from fitted curves. The kinetics of parthenin degradation in soil as modeled according to Equation 1 was compared further to a commonly used first-order kinetics model [$y=d$*exp(-kx) with k as the proportionality coefficient (Aldworth and Jackson 2008; Etzerodt et al. 2008)] in order to find the most appropriate model describing the degradation. All modeling was done with SPSS®, and the quality of curve fitting was assessed by F test for lack-of-fit based on an analysis of variance (P=0.05) or by graphical assessment of the distribution of data around regression lines. Comparison of curves was done by horizontal assessment (F test, P=0.05).

Once the concentration-response curves were fitted, the estimated DT_{50} (time where 50% of the applied toxin concentration disappeared) and b values (rate of degration) were used in a subsequent correlation analysis to quantify the significance of covariance with soil characteristics and experimental conditions. Quantification was done by the *Pearson* correlation coefficient r_P in case of a linear relation and the *Spearman* coefficient r_S in case of a nonlinear relation.

Results

The disappearance of parthenin from soil was characterized by a variable lag-phase period that ranged from a few hours after application up to several days, depending on experimental

conditions (Figs. 2, 4, 7). Comparison of HPLC-DAD chromatograms of treated soil after different incubation periods showed several unidentified metabolites, indicating that parthenin disappearance involves degradation of the compound (Fig. 1). Evaluation of the most appropriate model to describe the degradation showed that the first-order kinetic model failed to properly fit the data more frequently than the logistic model. In addition, the logistic model showed, on average of the various experimental conditions, smaller residual sum of squares, and was thus considered more adequate to describe parthenin degradation kinetics.

Degradation in Different Soils The DT_{50} values of parthenin differed significantly depending on the soil type used, while b values as a measure for the rate of degradation around DT_{50} did not (average b=1.99±0.17; mean ± standard error). Thus, degradation curves were merely shifted along the time axis (Fig. 2a). Parthenin disappeared most rapidly in the standard soil 3A with a DT_{50} of 43.1±5.3 h and, thus, significantly faster than in standard soils 5M and 2.1. Parthenin disappeared most slowly in standard soil 2.1 with a DT_{50} of 88.9±6.8 h, which was, however, not significantly different from standard soil 5M (DT_{50} 65.6±6.3 h).

Influence of Initial Substrate Concentration The initial soil parthenin concentration significantly influenced the rate of degradation (b), and the DT_{50} in CS soil, but curves did not significantly differ at 8 and 17 µg/g dry soil, 44 and 93 µg/g, or 425 and 820 µg/g (Fig. 2b). A significant positive correlation between initial parthenin concentration and b values of the curves as well as DT_{50} values also was observed (Fig. 3a). At the lowest concentration, the degradation of parthenin started after 9 h (DT_{10}=8.9±1.3 h), and 90% disappeared during the first nine days (DT_{90}=210.4±23.0 h). Increasing the initial parthenin concentration delayed the onset of degradation and led to a

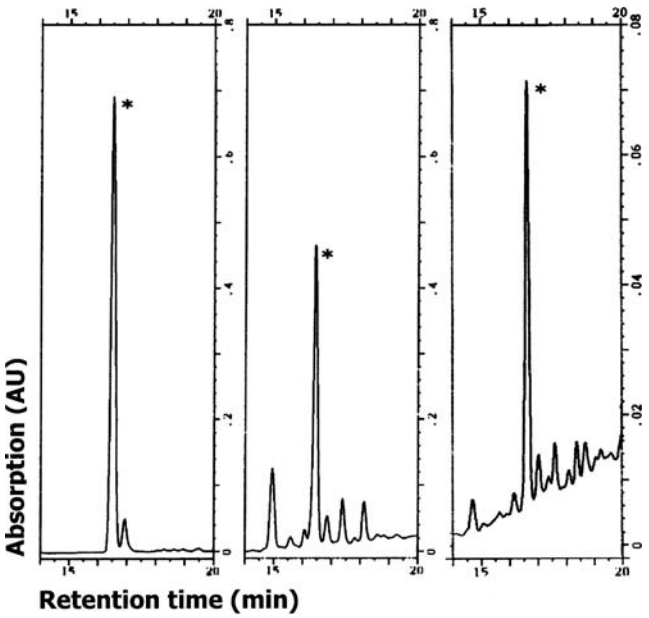


Fig. 1 HPLC-DAD chromatograms (220 nm) of compost soil samples incubated with parthenin at 1,000 µg/g dry soil for 1 h (*left*) or 16 d (*middle*) and of a natural infested soil sample (SA4; *right*); *= parthenin

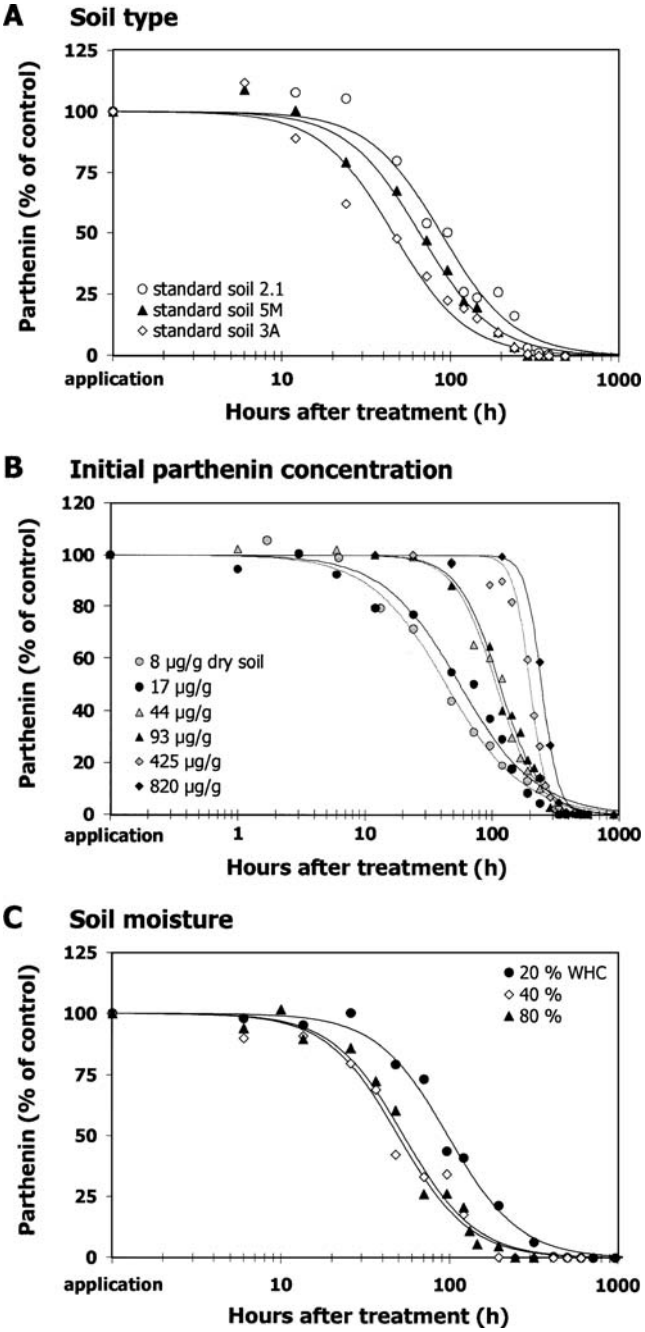


Fig. 2 Degradation of parthenin in soil as related to soil type (**a**; R^2= 0.96; see Table 2 for details), initial substrate concentration (**b**; R^2= 0.99), and soil moisture content (**c**; R^2=0.99); *WHC* water holding capacity; degradation in compost soil CS (except **a**) at 10 µg/g dry soil (except **b**), 40% WHC (except **c**), 20°C, and darkness

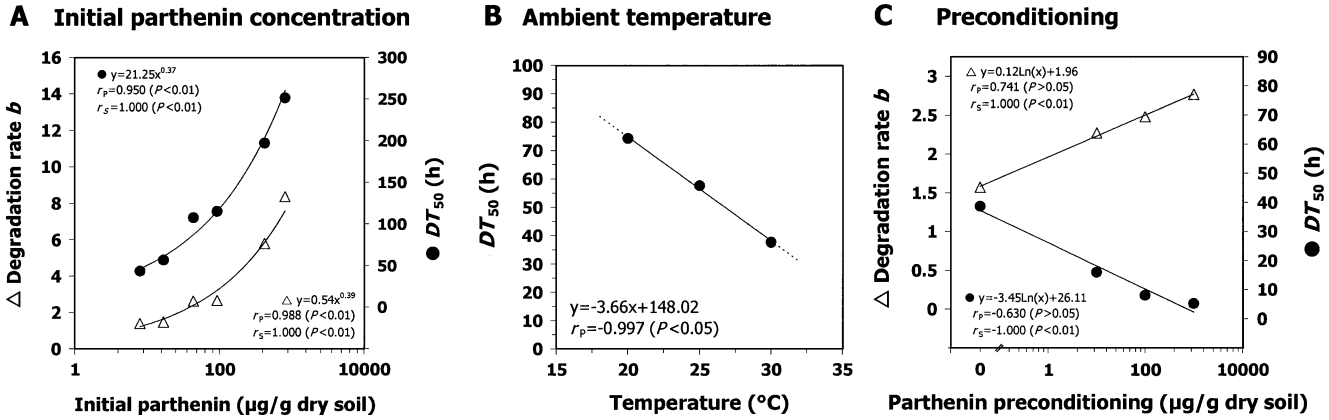


Fig. 3 Correlation of DT_{50}-values of parthenin or degradation rates *b* with initial substrate concentration (**a**), ambient temperature (**b**), and preconditioning (**c**); DT_{50} disappearance time 50%; *b* rate of change around DT_{50}; r_P *Pearson* correlation coefficient; r_S *Spearman* correlation coefficient; *P* probability level; degradation in compost soil CS at 10 µg/g soil (except **a**), 40% *WHC* water holding capacity, 20°C (except **b**), and darkness

prolonged lag-phase of about eight days at 820 µg/g (DT_{10}=193.1±9.1 h), which is nearly 22-fold later than at 8 µg/g. However, due to an increase in degradation rates with increasing concentration, 90% of the highest concentration disappeared during 14 days after application (DT_{90}=326.6±11.8 h), which is only less than 2-fold slower than at 8 µg/g. This showed that the degradation of parthenin is concentration-dependent and characterized by an expansion of the initial lag-phase with increasing concentration followed by an accelerated rate of degradation.

Influence of Soil Moisture Soil water content only significantly influenced the degradation of parthenin under dry conditions, with curves not differing at 40 and 80% WHC (DT_{50}=48.7±0.2 h at 40%; 53.9±0.2 h at 80%) (Fig. 2c). Degradation at 20% WHC was on average almost 2-fold slower than at the higher water contents, whereas the degradation rate was not significantly different at any soil moisture content (average b=2.05±0.12). As a consequence, DT_{50}-values did not correlate with soil moisture content (r_P=−0.689; P=0.516).

Influence of Ambient Temperature The incubation temperature had a significant influence on the disappearance of parthenin in the CS soil, whereas *b* values of the degradation curves did not (average b=1.80±0.11). Thus, degradation curves again were merely shifted along the time axis. The DT_{50} of parthenin was highest at 20°C (76.4±4.3 h) and decreased significantly by 1.3-fold at 25°C and 2.2-fold at 30°C. The observed negative correlation between incubation temperature and DT_{50}-values was significant at P=0.05 (r_P=−0.997) (Fig. 3b).

Influence of Soil Sterilization Sterilization of the CS soil prior to application of parthenin significantly delayed the onset of degradation and increased the DT_{50} of parthenin despite accelerating the rate of degradation. Compared to unsterilized soil (DT_{50}=69.6±5.4 h), sterilization led to a 5-fold slower degradation for the single sterilization treatment and a 9-fold slower degradation for the double treatment (Fig. 4a). The degradation curves of both sterilized soils showed on average 2-fold higher rates of degradation (*b*) as the unsterilized soil (b=1.41±0.15). Soil sterilization, therefore, enhanced the soil persistence of parthenin considerably.

Influence of Soil Preconditioning A subsequent degradation of 10 µg/g dry soil after preconditioning of CS soil with three different concentrations of parthenin showed a pronounced preconditioning effect (Fig. 4b). Parthenin degraded the slowest in the absence of a preconditioning (DT_{50}=38.7±2.0 h), more than 2-fold faster at a preconditioning of 10 µg/g, more than 4-fold faster at 100 µg/g, and more than 7-fold faster at 1,000 µg/g. Accordingly, there was a negative correlation between DT_{50}-values and preconditioning concentration (Fig. 3c). A preconditioning with parthenin steepened the degradation curve, although differences in *b*-values were not significant. Nonetheless, the observed nonlinear correlation between degradation rates (*b*) and preconditioning concentration was significant (Fig. 3c). Due to the steepening of the curves, 90% of the applied parthenin at 1,000 µg/g disappeared within 9.1 h after the onset of degradation (DT_{10}=2.3±0.3 h), while it took 147.2 h in the absence of a preconditioning after a lag-phase of 9.6 h. This showed that parthenin can disappear more rapidly from preconditioned soils.

Influence of P. hysterophorus Infestation Considering the observed preconditioning effect under laboratory conditions, soils infested with *P. hysterophorus* should be preconditioned

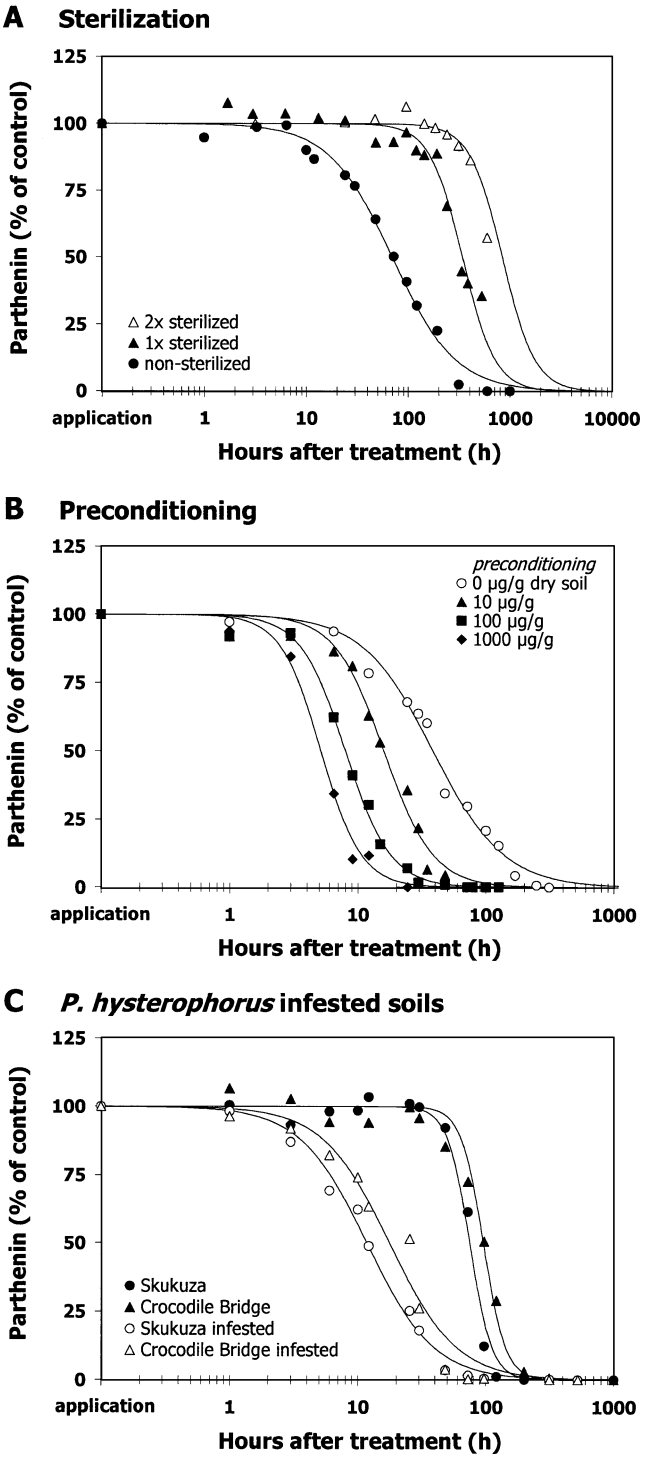


Fig. 4 Degradation of parthenin in soil as related to preceding sterilization (**a**; R^2=0.96), soil preconditioning with different parthenin concentrations (**b**; R^2=0.99), and natural infestation with *Parthenium hysterophorus* of two South African soils (location Skukuza and Crocodile Bridge) (**c**; R^2=0.97); degradation in compost soil (except **c**) at 10 µg/g soil, 40% *WHC* water holding capacity, 20°C, and darkness

for parthenin degradation if parthenin is actually released from plant material. Investigation of the degradation of parthenin in two naturally infested soils (SA1 and SA4) in comparison with adjacent, uninfested soils (SA2 and SA3) showed that degradation curves of both infested and uninfested soils did not significantly differ. However, parthenin, based on DT_{50}-values, was degraded 6-fold faster in the infested soil samples SA1 and SA4 (average DT_{50}= 14.4±1.1 h) (Fig. 4c). The degradation curves of the infested soils were further characterized by 3-fold lower rates of degradation (average b=1.66±0.15). Prior to the start of the experiment, chemical analysis of soil samples showed traces of parthenin in the infested soils (0.77±0.09 µg/g dry soil on average; Fig. 1), while no parthenin was detectable in the uninfested soils. After two weeks of preincubation under test conditions, no parthenin, however, was detectable in the infested soils either.

Correlation of Soil Characteristics with Degradation Investigation of the relationship between soil characteristics (Table 2) and DT_{50} values of the eight different soils showed a highly significant negative correlation for potential cation exchange capacity (CEC_{pot}; r_P=−1.000, P<0.001) and the clay content of the soils (r_P=−0.998, P=0.007) (Fig. 5). Correlations between DT_{50} values and the remaining soil characteristics as given in Table 2 were not significant (P> 0.05). This indicated that parthenin degradation is favored in clayey soils with high CEC_{pot}.

Soil Phytotoxicity Studies The phytotoxicity of parthenin in a soil substrate was studied by using the South African soil samples from *P. hysterophorus* infested (SA1 and SA4) and uninfested sites (SA2 and SA3). The bioassay showed that parthenin was more phytotoxic in the uninfested soils. However, the concentration-response curves of both infested and uninfested soils did not significantly differ (Fig. 6a). The uninfested soils showed on average a 4-fold higher parthenin phytotoxicity (EC_{50}=153.7±11.1 µg/g dry soil) than the infested soils (629.9±34.7 µg/g), and concentration-response curves were significantly shallower (average $b_{uninf.}$=1.57± 0.13, $b_{inf.}$=2.51±0.29). Accordingly, at the EC_{10} response level, 7-fold higher amounts of parthenin were needed in the infested soils (average EC_{10}=37.8±6.0 µg/g).

Soil Phytotoxicity over Time The question of whether parthenin remains bioavailable in a soil environment was investigated by monitoring the decline of the phytotoxic effect at 700 µg/g in CS soil. This concentration level nearly caused a 50% inhibition of plant growth in CS soil in a preliminary bioassay. The chemical analysis of parthenin degradation revealed an onset at 10.4±0.6 days (DT_{10}), a DT_{50} of 12.7±0.3 days, and 90% disappeared within 15.6± 0.9 days. Bioassay results showed that the observed initial

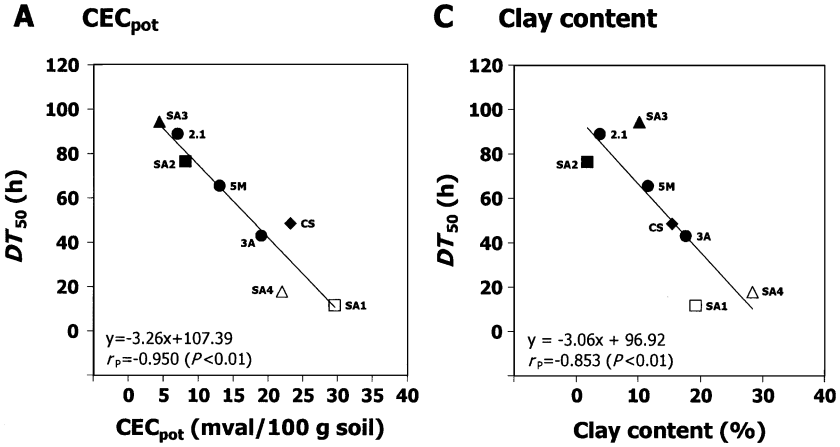


Fig. 5 Correlation of DT_{50}-values of parthenin with soil characteristics (see Table 2 for soil labeling); DT_{50} disappearance time 50%; CEC_{pot} potential cation exchange capacity; r_P *Pearson* correlation coefficient; *P* probability level; degradation at 10 µg/g soil, 40% *WHC* water holding capacity, 20°C, and darkness

inhibition by parthenin (46% of control) persisted for about 1.1 days (90% response level) before it declined. Stimulatory effects appeared from about 4.1 days (1% response level) after the start of the experiment. The stimulatory effect proved significant and reached a maximum of 191% of control at 10.5 days after application. As the chemical analysis estimated the onset of degradation at 10.4 days after application, the observed earlier decline in phytotoxicity and the induction of hormetic effects could not be caused by degradation of the active compound (Fig. 6b).

Soil Accumulation Studies The periodic application of parthenin at 400 µg/g again showed a pronounced and significant preconditioning effect along with a significant change in degradation rates at the second and third application (b_1=14.89±1.81; average $b_{2/3.}$=2.61±0.15) (Fig. 7). Parthenin was degraded with a DT_{50} of 210.5± 1.7 h for the first application, degraded more than 6-fold faster for the second application, and more than 13-fold at the third application. For the last application, the degradation started as early as 6.1 h after application (DT_{10}), and 90% had disappeared within 37.6 h. There was no indication for an accumulation of parthenin to biologically active levels in the CS soil, as the lower limits (C) of the three degradation curves did not significantly differ. Thus, at each application, parthenin was degraded to concentrations below the detection limit (<0.07 µg/g dry soil).

Discussion

After phytotoxins have been released into soils, the expression of their phytotoxicity can be significantly modified by physical, chemical, and biological soil properties. Testing the hypothesis of a compound's involvement in allelopathic interactions, therefore, requires an examination of its fate and persistence in soil, and soil concentrations

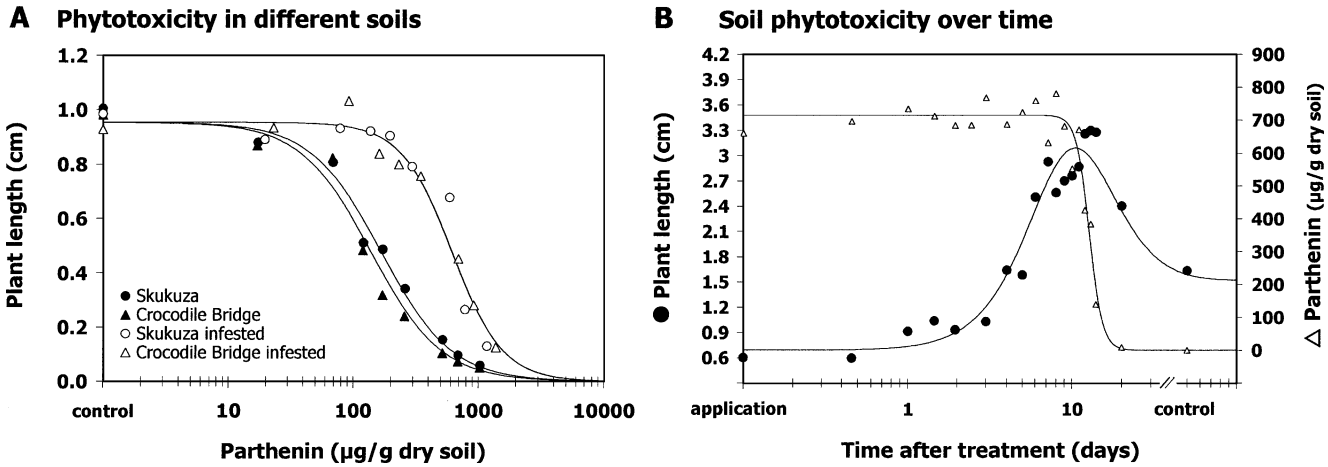


Fig. 6 Phytotoxicity of parthenin in soil as measured by total plant length (root + shoot) of *Lactuca sativa* as related to initial substrate concentration and soil type (**a**; R^2=0.88) or incubation period of 700 µg/g dry soil of parthenin in compost soil CS (**b**; $R^2_{bioassay}$=0.72; $R^2_{degradation}$=0.94); bioassay at 120% *WHC* water holding capacity, 24/18°C and 50 µE/m^{2}s for 12/12 h (**a**, **b**); degradation at 40% WHC, 20°C, and darkness (**b**)

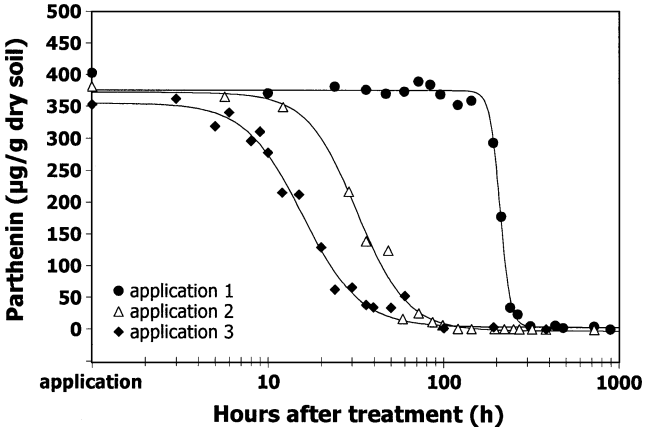


Fig. 7 Degradation of parthenin in compost soil CS after recurrent application of 400 µg/g soil at a time interval of 38 days each (20°C; darkness; 40% *WHC* water holding capacity); R^2=0.99

must be proven to adversely affect the receiving plant. Despite a promising allelopathic potential in the lab, short half-lives and very low field levels of major phytotoxins often deny an involvement in allelopathic interference and let other mechanisms of interference come to the fore. A recent example for this is the strong evidence refuting (–)-catechin involvement in the invasiveness of *Centaurea stoebe* L. (Duke et al. 2009a, b), despite previous reports (Bais et al. 2003; Inderjit et al. 2006; Perry et al. 2007). The investigation of the persistence of parthenin in soil revealed that parthenin also is quickly degraded with a mean DT_{50} of 59 h in six uninfested soils under standard experimental conditions. Abiotic and biotic variables proved to significantly impact parthenin degradation. Notably, preconditioning could reduce the observed standard DT_{50} value to nearly 5 h. Therefore, parthenin is considered to have rather low persistence in soil.

Regarding the inherent physico-chemical characteristics of soils, parthenin turned out to be degraded faster in clayey soils with high CEC_{pot}. The importance of soil characteristics in degradation of allelochemicals is well known (Dalton et al. 1989; Kobayashi et al. 2004), and selected soil characteristics also have been shown to be correlated closely to mineralization, degradation, and/or sorption/desorption of certain commercial pesticides (Sparling et al. 1998; Olvera-Velona et al. 2008). In the case of parthenin, CEC_{pot} proved to be indicative of the capacity of soils to degrade parthenin. The primary factors that determine CEC_{pot} are the amount and type of clay and soil organic matter content (Scheffer and Schachtschabel 2002). In this study, a significant correlation between DT_{50} of parthenin and clay content of soils, but not C_{org} was observed. Calvet et al. (1980), however, pointed out that the correlation between the degradation of non-ionic herbicides and soil organic matter is not always very good across the range of 0 to 4% organic matter. This range includes all but one of the soils tested in this study, so the soils used might have contained too little C_{org} for a significant correlation. As parthenin proved more stable in certain soil types, it might be expected that *P. hysterophorus* could especially invade these soil types if parthenin-mediated allelopathy were operative. Such evidence, however, does not exist under natural conditions, as *P. hysterophorus* preferably colonizes clay loam to clay soils, and is especially capable of out-competing other plant species on heavy cracking clays (Parthenium Weed Management 2004). Thus, *P. hysterophorus* seems to preferably and most efficiently colonize soils that have a higher capacity to degrade parthenin.

The influence of initial substrate concentration supports the graphically derived assumption that parthenin degradation is not true first-order kinetics as DT_{50}-values did change with concentration. The same has been observed previously for other allelochemicals (Borek et al. 1995; Etzerodt et al. 2008), and is suggested to apply for most current-generation agrochemicals (Aldworth and Jackson 2008). Degradation of higher initial parthenin concentrations proved to be characterized by a broad lag-phase preceding the onset of degradation. This may indicate a possible involvement of microorganisms, as high concentrations may cause paralysis or toxicity to soil microorganisms and, thus, result in a delayed onset of degradation due to required adaptation and acclimation (Bending and Rodriguez-Cruz 2007; Etzerodt et al. 2008). Despite the observed prolonged lag phase, higher concentrations completely disappeared just less than 2-fold more slowly than lower concentrations. Parthenin-mediated plant interactions may thus be rather short-lived even if high amounts of parthenin are released.

Manipulation of soil moisture showed no significant influence between 40 and 80% WHC. However, the degradation of parthenin was delayed significantly at 20% WHC, which may be a further indication for the involvement of microorganisms, as their activity significantly decreases under dry conditions (Schricker 1996). The optimum soil moisture content for microorganism activity ranges between 40 to 70% WHC (Schricker 1996; Terytze et al. 2000), which would explain the observed lack of correlation between the tested soil moisture levels and DT_{50} values of parthenin.

Ambient temperature was significantly correlated with parthenin disappearance in terms of a faster degradation with increasing incubation temperature. A fast, temperature-sensitive disappearance of parthenin can, therefore, be expected to occur in the tropic and subtropic environments under which *P. hysterophorus* flourishes, albeit with seasonal fluctuation. As most microorganisms are mesophilic and promoted by increasing temperatures within a certain

temperature range (Scheffer and Schachtschabel 2002), the observed temperature dependence may further support the assumption of a microbial involvement.

Additional evidence for a microbial degradation could be obtained by a significantly declined degradation in sterilized soils and an enhanced degradation at repeated applications. This is consistent with findings of a microbial transformation of parthenin (Bhutani and Thakur 1991; Abdel Halim et al. 2007), and it points to the possibility of enriching soils with microorganisms that are capable of degrading parthenin, a phenomenon that is well known from several commercial herbicides (Walker et al. 1993; Pussemier et al. 1997). Hence, it was no surprise that soil samples previously infested with *P. hysterophorus* showed on average a 6-fold higher capacity to degrade parthenin than adjacent, uninfested soils. This along with the quantifiable presence of parthenin in both infested soil samples showed that parthenin must have actually been released by *P. hysterophorus* plants or plant residues under field conditions and degraded in the soil beneath the sampled stands. Therefore, the observed low persistence of parthenin in these soils might have resulted from a natural preconditioning for parthenin degradation. On the other hand, DT_{50} values of the infested and adjacent soil samples both followed the observed correlation to CEC_{pot} and clay content. This raises the question of which aspect is more decisive for parthenin degradation: a preconditioning effect or physico-chemical soil factors? Degradation studies with pesticides have shown that for naturally faster degrading soils, an enhancement of biodegradation by repeated applications is small or negligible (Bischoff et al. 2005). However, our results showed that degradation curves in soils that differed in physico-chemical soil factors were merely shifted along the time axis, while degradation rates b also changed in the case of preconditioning or repeated application, and *P. hysterophorus* infestation. Hence, physico-chemical factors alone do not explain observed degradation kinetics of infested soils. Rather, the fate of parthenin in soil is most likely governed by physico-chemical processes combined with microbial activity, whereas the importance of the particular process may fluctuate depending on the situation.

Apart from this, it is difficult to find adequate soils for use as 'control soils'. This study used soil samples from adjacent, uninfested sites as control, which is a common practice in allelopathy research (Weidenhamer et al. 1989; Kohli and Batish 1994). Analysis showed, however, that the current 'control soils' differed markedly in soil characteristics. Additionally, allelopathic plant infestation may further cause changes in soil characteristics (Kohli and Batish 1994; Inderjit 1998), and thus it is obvious that the role of infestation is difficult to prove with such differing soils. The isolation of microorganisms capable of degrading parthenin and the possibility of enrichment by parthenin may shed light on this. A knowledge of the spectrum of microorganisms that degrade parthenin may further help to explain the fact that we found indications for a biodegradation in South African and German soils that could be expected to differ in their spectrum of microorganisms.

As our results indicated the formation of several, unidentified metabolites, we considered the possibility that parthenin could be degraded to a more toxic compound. Concentration-response curves for the inhibition of *L. sativa* by parthenin in soil revealed a higher phytotoxicity in uninfested soil samples that showed a higher persistence of parthenin. Thus, the phytotoxicity of parthenin is impaired if degraded faster, i.e., in soils with high CEC_{pot}/clay content and/or preconditioned for parthenin degradation. A possible explanation for this is that parthenin is transformed to metabolites of lower or no phytotoxicity. A parthenin-mediated allelopathic effect might, therefore, be based on parthenin as the primary allelochemical and require high soil levels or a periodic exposure to compensate for rapid degradation. Evidence for such high soil levels were not found in the current study. In five soil samples taken at three different times (August 2005, November 2006, October 2007) at two sites in the KNP with high *P. hysterophorus* infestations, we measured parthenin levels between 0.1 and 6.4 µg/g dry soil, even though one sampling event was in early August, a time when most *P. hysterophorus* leaf residues are expected on the soil surface (Weeds of National Significance 2003). These soil concentrations are 6–2628 times lower than the currently observed EC_{10} values in bioassays, the virtual 'no observable effect levels'. Thus, quantified levels for the KNP soils are too low to deduce a significant impact on neighboring plants.

In addition, a comparison of the phytotoxic effect in soil over time with the respective degradation curve showed that the onset of degradation lags behind the loss in phytotoxicity. A plausible explanation for this is that parthenin does not remain fully bioavailable in the soil. The high recovery rates during the lag-phase showed, however, that inactivation obviously does not provide protection from the applied acetone/TBME extraction. Hence, the estimated soil concentration should represent both the biologically available and unavailable fraction of parthenin. The exposure of recipient plants to parthenin in the infested KNP soils is, thus, even lower than currently determined. A lowering of the bioavailability further induced growth stimulatory effects. Hormetic effects at low concentrations are well known for parthenin from pure compound studies (Belz et al. 2007; Belz 2008) and plausibly such effects would also appear if parthenin concentrations in soil were lowered by inactivation. Hence, the phytotoxicity of parthenin in soils is impaired by a time-dependent decline in bioavailability and the induction of hormesis. A comparison of the concentration-response

values obtained with a single parthenin application with concentrations that occur under natural field conditions, however, is not straightforward, as such a comparison does not account for temporal allelochemical dynamics (Inderjit et al. 2006; Perry et al. 2007). As our samplings consistently detected low levels of parthenin, this issue may be relevant here, and future studies that account for a periodic exposure of recipient plants are required.

Finally, we wanted to determine whether or not parthenin accumulates in soils over time to levels sufficient to affect the development of other plants. Other compounds such as juglone or phenolic acids can accumulate in soil through a mechanism of reversible sorption (Cecchi et al. 2004; von Kiparski et al. 2007). The triple application of parthenin at 400 µg/g soil, however, provided no indication that parthenin accumulates in soils.

In summary, although previous studies assigned parthenin a role in the invasion success of *P. hysterophorus*, this study revealed several aspects that hamper the extent to which parthenin is involved in allelopathy of *P. hysterophorus*. Parthenin was rather quickly degraded without any evident accumulation to toxic levels over time. Schmidt (1988) and Schmidt and Ley (1999) claimed that a rapid bioinactivation of suspected allelochemicals in natural soils makes it unlikely that these compounds are important mediators of plant–plant interactions under natural conditions. Furthermore, inactivation and degradation of parthenin can lead to hormetic growth stimulation. A soil-based allelopathic activity mediated by parthenin may, thus, be rather short-lived and/or hormetic without significant residual activity. The chemical control of another plant will require the release of high amounts of parthenin. High or biologically active soil concentrations of parthenin were not demonstrated in our analysis of infested soil samples. Finally, *P. hysterophorus* seems to be particularly invasive and capable of outcompeting other plant species under soil and environmental conditions that favor the degradation of parthenin. Based on this, we conclude that the present state of research provides insufficient evidence to support the hypothesis that parthenin contributes to the invasiveness of *P. hysterophorus*.

On the other hand, given that parthenin-containing leaf leachates of *P. hysterophorus* stimulate plant chlorophyll content (Kumari and Kohli 1987), one can speculate that self-stimulatory allelopathy rather than inhibitory allelopathic interactions may contribute to the capacity of *P. hysterophorus* to form dense pure stands. Self-stimulating effects are presumed to be important for the dominance of a species in a natural habitat (Schlee 1992) and may, thus, be a key process involved in altering the balance among species. Furthermore, a periodic parthenin input from both live plants and decaying plant residues into soil and/or the contribution of other allelochemical(s), could still conceivably produce an allelopathic effect. Bioassay-guided isolation identified parthenin as the leading putative allelochemical of *P. hysterophorus*, but additionally several phenolic acids and other plant constituents as minor inhibitors (Kanchan and Jayachandra 1980). Allelopathy of *P. hysterophorus* could possibly involve a complex mixture of compounds that act additively or synergistically. Tharayil et al. (2008) recently showed that phenolic acids significantly extended the persistence and bioavailability of companion compounds in allelochemical mixtures. Companion compounds could inhibit microbial growth and diminish populations able to degrade parthenin. Thus, an allelopathic hypothesis cannot be conclusively rejected. That said, the weed is extremely effective at invading disturbed areas with poor groundcover (e.g., overgrazed pastures, wastelands) or bare sites with exposed soil (e.g., roadsides, stockyards, watering points) (Weeds of National Significance 2003; Parthenium Weed Management 2004) and does not usually establish in or displace undisturbed vegetation. The latter would be expected if allelopathy is operative in plant succession (Rice 1977). Any *P. hysterophorus* potential allelopathic effects may, therefore, be of secondary importance in consideration of the plant's other highly competitive features such as a large and persistent seedbank, a fast germination rate, a short life cycle, a high fecundity, multiple generations per year, a biennial capacity, and an efficient use of resources.

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lipid layer, cuticular hydrocarbons (CHCs) are universally present (Lockey 1988) and are particularly numerous in social insects, especially the ants, which makes CHC profile analysis problematic (Martin and Drijfhout 2009b). Ants often are referred to as chemical factories since they are known to posses over 50 distinct exocrine glands (Billen 2004), the majority of which are active in pheromone production (Billen and Morgan 1998). The availability of gas chromatography (GC) with flame ionization detection in the late 1950s, and more recently GC-mass spectrometry (GC-MS), has made CHC studies accessible to biologists. In insects, studies on non-hydrocarbon signals, such as recognition proteins that are the focus of much vertebrate research (Hurst et al. 2001), remain an important but peripheral field (Dapporto et al. 2008; Turillazzi et al. 2007). In ants, the focus on CHCs is well founded, as the nest-mate discrimination systems in several ant species are known to be based on hydrocarbons (Bonavita-Cougourdan et al. 1987; Yamaoka 1990; Lahav et al. 1999; Akino et al. 2004; Martin et al. 2008a; Greene and Gordon 2007; Guerrieri et al. 2009), and these compounds are known to be perceived by ants (e.g., Ozaki et al. 2005).

The first ant CHC profile was reported in 1970 (Martin and MacConnell 1970). Since then, CHC profiles for over 80 ant species have been published, and the number continues to grow rapidly. Although there have been various reviews on the roles of semio-chemicals, including CHCs in social insects (Bradshaw and Howse 1984; Lenoir et al. 1999, 2001a, b; Howard and Blomquist 2005), none has comparatively categorized the various CHCs described across these studies. The aim of this review is to compare the various CHCs described from 1970 to 2007 across 78 ant species from five different subfamilies. We looked for patterns that may reveal some general insights into the roles of ant CHCs.

Methods and Materials

A literature search was conducted to identify published papers from 1970 through 2007 on ant species whose CHCs were analyzed in detail. These included studies on the postpharyngeal gland (PPG), an exocrine gland unique to ants that is involved in pheromone communication (Soroker et al. 1995; Oldham et al. 1999). The CHC profiles of the PPG, and the cuticle are always similar (Bagnères and Morgan 1991; Soroker et al. 1995; Akino et al. 2004). However, studies on the contents of the Dufour's gland were excluded, as these glands contain trail and alarm pheromones, as well as CHCs (Regnier and Wilson 1968; Morgan et al. 2003). The various studies used differing extraction methods; e.g., solvent extraction with hexane or pentane, solid phase microextraction (SPME), and solid sampling (Morgan 1990). The GC conditions (non-polar columns with either 100% or 95% methyl-silicon) and temperature profiles (50/70°C to 300/350°C) were similar across the majority of studies. However, there was no conformity on the level of analysis, which has improved greatly over time, or how the compounds were reported. Across the studies, only 44% reported CHCs proportions; 8% of these studies placed the CHC in arbitrary groups (e.g., >1%, >10%), while the remaining 48% of the studies simply reported compounds as present or absent. A further complication is that only about half of the studies attempted to determine positional isomers. Due to this variation in data quality, and the vast quantity of data collated, we restricted our review to the presence (indicated by 'X' in Table 1) or absence of each compound irrespective of the chain-length. The chain-length data for each CHC (not shown) was used to calculate the total number of compounds and ratio of single compounds vs. homologous series. A hierarchical cluster analysis of all CHCs (present or absent) was performed using the statistical package for social sciences (SPSS v14; Field 1970) with squared Euclidean distances with average linkage (between groups).

Results and Discussion

Across the 78 species, almost 1,000 CHCs have been described (Table 1). These are composed of 187 distinct homologous series belonging to ten distinct hydrocarbon groups (Tables 1, 2), which are all built upon the same *n*-alkane backbone with 1, 2, or 3 double bonds or the inclusion of 1, 2, 3, or 4 methyl groups. Among the 78 species, the sequence of occurrence was as follows: *n*-alkanes > monomethylalkanes > dimethylalkanes > alkenes > dienes>> trimethylalkanes>> methylalkenes > methylalkadienes > trienes > tetramethylalkanes (Table 2). As the CHC structures become more complex, fewer species produce them, e.g., 73 species produced methylalkanes, while only one produced tetramethylalkanes (Fig. 1). Furthermore, more complex CHC structures are usually produced by species that already produce the more simple structure, except in three rare cases (*Atta colombica*, *Pachycondyla apicalis*, and *Camponotus fellah*) (Table 1, Fig. 1).

n-Alkanes were found in all 78 species except in the queens of *Formica gnava* and *F. occulta* (Table 1). Odd-numbered C_{25} to C_{35} *n*-alkanes typically dominate ant CHC profiles, as is almost universally the case in insects (Lockey 1988; references cited herein). Odd-numbered *n*-alkanes also are characteristic components of epicuticular waxes produced by terrestrial higher plants (McDuffee et al. 2004). In insects, the primary function of CHCs is to act as anti-desiccation and waterproofing compounds (Edney 1977), and their abundance is correlated with varying environmental conditions (Wagner et al. 2001; Martin and

Table 1 A comparison of the types of hydrocarbons extracted from the cuticle or postpharyngeal glands of ants (Hymenoptera: Formicidae)[a]

	alkanes	monomethylalkanes																						alkenes							dienes	tri	methylalkenes	methyldienes	dimethylalkanes																		
1st position	n	19	17	15	13	11	9	7	5	3	20	18	16	14	12	10	8	6	4	2	?	12	11	10	9	8	7	?		?	?	?	2						3												
2nd position																												?		?		?	6	8	10	12	14	16	5	7	8	9	11	13	14	15	16	17	18	19
3rd position																														?																				
4th poistion																																																			
Acromyrmex subterraneanus	x		x	x	x	x								x	x	x																																		
Atta colombica	x																																																	
Formicoxenus nitidulus	x				x	x	x	x	x	x					x						x							x																						
Formicoxenus provancheri	x		x	x	x	x	x	x	x	x				x	x	x			x		x				x																								
Formicoxenus quebecensis	x			x	x	x	x	x	x	x				x	x	x					x				x			x																			x	x	x	
Harpagoxenus sublaevis	x				x	x	x	x	x	x											x																												
Leptothorax acervorum	x			x	x	x		x	x	x					x				x		x				x			x																						
Leptothorax gredleri	x				x	x	x			x					x																x																				
Leptothorax kutteri	x					x				x															x			x																						
Leptothorax muscorum	x					x	x	x	x	x																																								
Leptothorax nylanderi	x		x	x	x	x	x	x	x	x				x	x	x	x	x	x									x																						
Myrmicaria eumenoides	x									x											x[b]	x		x	x	x	x	x[b]																						
Manica rubida	x				x	x	x	x	x	x																																								
Myrmica alaskensis	x			x	x	x	x	x	x	x				x	x	x					x				x			x																						
Myrmica incompleta	x		x	x	x	x	x	x	x	x				x	x	x			x		x				x			x																						
Myrmica rubra	x			x	x	x	x	x	x	x				x	x	x	x	x																																
Pognomyrmex barbartus	x		x	x	x	x	x	x	x	x				x			x			x																													
Aphaenogaster senilis	x				x	x	x	x	x	x					x	x	x	x	x		x																													
Messor barbarus	x			x	x	x	x	x	x	x				x	x	x	x	x	x																															
Tetramorium bicarinatum	x			x	x	x	x	x	x	x				x	x	x		x			x							x																						
Solenopsis invicta	x				x					x																																								
Wasmannia auropunctata	x			x	x	x	x	x	x	x									x		x							x																						
Ectatomma ruidum	x		x	x	x	x	x		x	x					x	x									x		x	x	9,14																				
Gnamptogenys striatula	x				x	x	x			x	x	x		x	x				x		x																													
Platythyrea punctata	x				x	x	x	x	x	x					x	x				x					x		x	x	2,9; 8,16																				
Diacamma ceylonese	x			x	x	x	x	x	x	x				x	x	x	x		x		x																													
Dinoponera quadriceps	x		x	x	x	x	x	x	x	x				x	x										x			x																						
Harpegnathos saltator	x	x	x	x	x	x	x	x		x			x	x	x				x			x			x																									
Pachycondyla apicalis	x			x	x	x		x		x																		x	2,9																					
Pachycondyla goeldi	x					x															x							x																						
Pachycondyla inversa	x					x	x	x	x	x					x	x		x	x	x	x																													
Pachycondyla villosa	x	x	x	x	x	x	x	x	x	x				x	x	x	x	x	x	x	x												x																	
Myrmecia gulosa	x			x	x	x	x			x				x	x	x					x																													
Iridomyrmex purpureus	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x			x		x				x																								
Iridomyrmex nitidiceps	x			x	x	x		x		x	x		x	x	x	x			x		x				x	x																							
Linepithema humile	x	x	x	x	x	x	x	x	x	x				x	x	x			x		x				x	x	x																						
Nothomyrmecia macrops	x			x	x	x	x	x	x	x					x	x	x	x	x						x						x[c]		x[c]																
Camponotus fellah	x			x	x	x		x		x							x		x		x																													
Camponotus floridianus	x			x	x	x	x	x		x		x	x	x	x	x			x																														
Camponotus vagus	x			x	x	x	x	x		x				x	x	x	x		x	x	x																												
Cataglyphis bombycinus	x			x	x	x	x	x	x	x				x	x		x								x																							
Cataglyphis cursor	x			x	x	x		x	x	x						x			x				x	x	x																							
Cataglyphis floricola	x		x	x	x	x	x	x	x	x				x	x	x		x	x	x	x				x		x																						
Cataglyphis hispanicus	x		x	x	x	x	x	x	x	x					x	x			x	x	x					x		x																				
Cataglyphis humeya	x			x	x	x	x	x	x	x																x		x				x																	
Cataglyphis iberica	x			x	x	x	x	x	x	x									x							x																							
Cataglyphis ibericus	x		x	x	x	x	x	x	x	x				x	x	x		x	x	x	x	x	x	x	x																							
Cataglyphis niger	x			x	x	x	x	x	x						x																																		
Cataglyphis rosenhaueri	x		x	x	x	x	x	x	x	x			x	x	x	x		x	x	x	x				x		x		x																				
Cataglyphis velox	x		x	x	x	x	x	x	x	x				x	x				x		x				x			x																				
Formica aquilonia	x		x	x	x	x	x	x	x	x				x	x	x	x				x																												
Formica candida	x		x	x	x	x	x	x	x	x											x							x																						
Formica cinerea	x		x	x	x	x	x	x	x	x								x	x		x																												
Formica cunicularia	x		x	x	x	x	x	x	x	x				x	x	x			x			x			x		x																			x		
Formica exsecta	x		x	x	x	x	x	x	x																x																							
Formica fusca	x		x	x	x	x	x	x	x	x					x	x	x	x	x		x																												
Formica glacialis	x			x	x	x				x											x																												
Formica gnava Q					x	x	x								x	x				x																													
Formica japonica (type II)	x			x	x	x	x												x						x		x																						
Formica lemani	x			x	x	x	x	x	x	x															x																							
Formica lugubris	x		x	x	x	x	x	x	x	x				x	x	x	x				x																												
Formica montana	x																				x																												
Formica occulta Q					x	x	x	x						x	x	x	x	x	x	x																													
Formica polyctena	x		x	x	x	x	x	x	x	x				x	x	x	x				x																												
Formica pratensis	x		x	x	x	x	x	x	x	x				x	x	x	x				x																												
Formica rufa	x		x	x	x	x	x	x	x	x				x	x	x	x				x																												
Formica rufibarbis	x		x	x	x	x	x	x	x	x				x	x	x			x																													
Formica sanguinea	x		x	x	x	x	x	x	x	x				x	x	x	x		x		x																												
Formica selysi	x				x	x	x		x	x															x			x	9,19; 9,21; 9,23																				
Formica truncorum	x		x	x	x	x	x	x	x	x				x	x	x	x								x																							
Formica uralensis	x		x	x	x	x	x	x	x	x				x	x	x	x				x																												
Lasius fuliginosus	x			x	x	x	x	x	x	x									x						x																							
Lasius sakagamii	x			x	x	x	x																		x																							
Lasius niger	x	x	x	x	x	x																			x																							
Polyergus breviceps Q	x																			x																												
Polyergus rufescens	x			x	x	x	x	x	x	x				x	x	x			x			x		x		x																						
Proformica longiseta	x			x		x	x		x	x						x	x	x	x																													
Rossomyrmex minuchae	x			x		x	x		x	x						x	x	x	x																													

[a] Subfamilies are separated by dashed lines.

[b] *M. eumenoides* an additional five alkenes (16,14,5,4) and ten dienes (5-19,-21,-23,-25,6-9,7-15,-21,-23,-25,-27)

[c] *N. macrops* an additioanl six methlyalkanes and three methlydienes

Table 1 (continued)

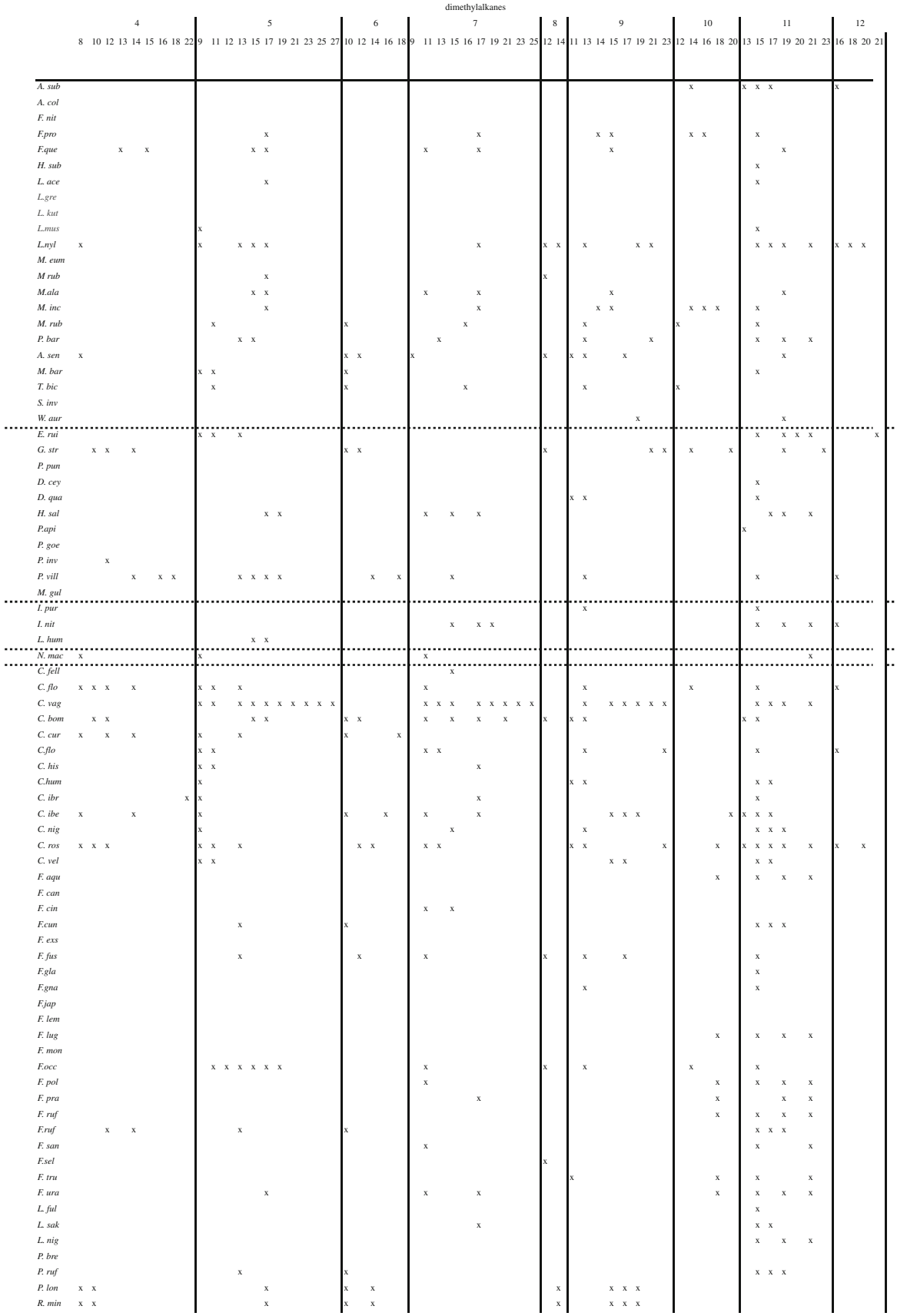

Table 1 (continued)

	dimethylalkanes																	trimethylalkanes																													tetramethylalkanes				References
	13							14					15					3				4			5			7							8			9			11		13				3		4	5	
	15	17	18	19	21	23	25	16	18	20	22	24	12	14	19	21	23	7	11	13	15	8	10	12	9	13	15	11	11	13	15	15	17	17	12	13	13	15	15	15	15	15	15	15	17	17	7	9	8	9	
																		11	15	17	17	12	14	16	13	17	19	15	21	21	19	21	21	23	16	17	23	19	21	23	19	21	19	21	21	23	11	15	12	13	
																																															15	21	16	17	
A. sub		x						x																																											Richard et al 2004
A. col																		x				x																													Martin & MacConnell, 1970
F. nit																																																			Martin et al 2007
F.pro		x																																																	Lenoir et al 1997
F.que																																																			Lenoir et al 1997
H. sub																																																			Kaib 1993
L. ace					x																																														Kaib 1993; Tentchert et al., 2002
L.gre																																																			Tentchert et al., 2002
L. kut																																																			Franks et al., 1990
L.mus																																																			Kaib 1993
L.nyl																																																			Trabalon et al 2000
M. eum																																																			Kaib et al 2000
M rub																																																			Bagneres et al 1991
M.ala																																																			Lenoir et al 1997, Lohman et al 2006
M. inc	x	x																																																	Lenoir et al 1997; Lohman et a., 2006
M. rub		x																																																	Bagneres and Morgan 1990
P. bar		x		x	x				x	x																															x	x	x	x	x	x					Wagner et al 1998, Nelson et al., 2001
A. sen																						x																													Lenoir et al 2001
M. bar																							x	x											x																Provost et al 1994
T. bic		x																							x																										Astruc et al 2001
S. inv	x																																																		Obin 1986; VanderMeer et al., 1989
W. aur																																																			Errard et al 2005
E. rui		x		x											x																																				Howard et al., 2001
G. str																			x																																Lommelen et al 2006
P. pun																																																			Hartmann et al 2005
D. cey		x																																																	Cuvillier Hot et al 2001
D. qua		x													x																																				Monnin et al 1998
H. sal		x		x	x	x					x	x			x																																				Liebig et al 2000
P.api																																																			Hefetz et al., 2001
P. goe																																																			Denis et al 2006
P. inv																																																			Tentschert et al 2001
P. vill		x		x		x	x		x						x	x	x									x																									Lucas et al 2004, D'Ettore and Heinze 2005
M. gul																																																			Cavill et al. 1970; Dietemann et al., 2003
I. pur		x																																																	Brophy et al., 1973; van Wilgenburg 2006
I. nit		x		x					x						x	x																									x				x						Brophy et al 1983
L. hum															x					x	x					x	x																								Liang et al 2001, Brophy et al., 1983
N. mac																																																			Vance Brown et al 1990
C. fell																															x																				Boulay et al 2000, Katzav Godansky 2004
C. flo		x																x				x						x																			x	x	x	x	Endler et al 2004
C. vag		x		x	x										x	x													x	x		x					x			x		x									Meskali et al 1995
C. bom	x																															x				x															Dahbi et al., 1996
C. cur																																																			Nowbahari et al., 1990
C.flo																																																			Dahbi et al., 1996
C. his																																																			Dahbi et al., 1996
C.hum																																																			Dahbi et al., 1996
C. ibr		x																																																	Dahbi & Lenoir 1998
C. ibe	x	x																									x				x					x		x													Dahbi et al 1996,1998, Dabhi and Lenoir 1998 a,b
C. nig																																																			Lahav et al. 2001
C. ros		x		x					x						x												x				x		x	x				x	x												Dahbi et al., 1996
C. vel		x													x																																				Dahbi et al., 1996
F. aqu	x	x																																																	Martin et al 2007
F. can																																																			Martin et al 2007
F. cin																																																			Martin et al 2007
F.cun		x		x											x																																				Bonavita Cougordon 1996, 1997
F. exs																																																			Martin et al 2007
F. fus																																																			Martin et al 2007; Hannonen et al., 2002
F.gla																																																			Lohman et al 2006
F.gna																																																			Johnson et al 2001
F.jap																																																			Akino et al 2004
F. lem																																																			Martin et al 2007
F. lug	x	x																																																	Martin et al 2007
F. mon																																																			Henderson et al., 1990
F.occ																																																			Johnson et al 2001
F. pol	x	x																																																	Martin et al 2007
F. pra		x																																																	Martin et al 2007
F. ruf		x																																																	Martin et al 2007
F.ruf		x		x											x																																				Bonavita Cougordon 1996, 1997
F. san	x	x																																																	Martin et al 2007
F.sel																																																			Bagneres et al 1991
F. tru		x																																																	Akino, 2006; Martin et al 2007
F. ura		x																										x																							Martin et al 2007
L. ful																																																			Akino 2002
L. sak																																																				Akino and Yamaoka 1998
L. nig	x		x										x	x		x	x																																		Akino and Yamaoka 1998
P. bre																																																			Johnson et al 2001
P. ruf		x		x											x																																				Bonavita Cougordon 1996, 1997
P. lon																																																			Errard et al 2006
R. min																																																			Errard et al 2006

Table 2 Summary of the major classes of cuticular hydrocarbons extracted from ant (Hymenoptera: Formicidae) species

Name	Number of compounds	Number homologous series	Frequency[a]
n-Alkanes	23	1	97%
Monomethylalkanes	194	19	96%
Dimethylalkanes	602	96	84%
Trimethylalkanes	48	29	22%
Tetramethylalkanes	4	4	1%
Alkenes	47[b]	10	73%
Dienes	51[b]	16	27%
Trienes	1	1	1%
Methylalkenes	17	7	4%
Dimethylalkenes	6	4	1%
TOTALS	993	187	

[a] Percentage of 78 species possessing each CHC group

[b] Positional isomers determined in only a few cases

Drijfhout 2009a). At ambient temperatures, *n*-alkanes heavier than C_{18} are solid; however, the addition of a double bond or methyl group into the alkane reduces their melting point by up to 50°C (Gibbs and Pomonis 1995a). Furthermore, while the melting points of pure CHCs occur over a narrow temperature range, mixtures of CHCs result in melting occurring continuously over a broad temperature range (Gibbs 1995b). This may help explain why monomethylalkanes are also present in all 78 species (except *F. montana* and *Atta colombica*). Like *n*-alkanes, theses compounds occur as homologous series (Table 2) of a variety of chain-lengths, but C_{19} to C_{33} *n*-alkanes are predominant and often comprise >50% of the total peak area of the entire profile. This combination of *n*-alkanes and monomethylalkanes will result in a waterproof layer that remains flexible since, when combined, they have a solid-liquid phase transition over a wide range of ambient temperatures (Gibbs 1995b). Some of the monomethylalkanes also may be used as chemical communication signals (Nelson 1993), but their ubiquity means that they could be general indicators only. They may also be linked to dimethyl production because in the ant *F. cinerea,* which produces large quantities (>30%) of 7,15-dimethylalkane, an unusually high proportion (>20%) of 7-monomethylalkane also was produced (Martin et al. 2008c). The ubiquity of *n*-alkanes, and their flexible response to environmental factors, makes it difficult to encode stable and distinctive signals that must be a prerequisite for species and nest-mate recognition. However, alkanes might be used to convey a simple message such as whether or not an ant is a forager (Greene and Gordon 2003; Martin and Drijfhout 2009a) or whether or not a queen is mated (Hora et al. 2008).

Dimethylalkanes are by far the most numerous group of compounds, with over 600 presently described (Table 1). They are species-specific, and can even be colony-specific (Martin et al. 2008b). These compounds are usually produced at one chain-length or as a short homologous series (Table 2), and are typically present in small quantities relative to *n*-alkanes and monomethylalkanes. As dimethylalkanes represent over half of all CHCs currently described in ants, it is not surprising that several studies have suggested that dimethylalkanes play an important role in recognition (Johnson et al. 2001; Endler et al. 2004; Martin et al. 2008b; Guerrieri et al. 2009). Furthermore, there are a vast number of possible dimethyl positions that potentially exist, but have yet to be described.

Only 13 species did not produce any dimethylalkanes (Table 1). Of these, 12 species produce alkenes, although monomethylalkanes and *n*-alkanes were still produced. Double bond positions have been determined in around half of the studies showing that the odd positional isomers dominate (*Z*9, 33 spp; *Z*7, 11 spp; *Z*11, 4 spp. vs. *Z*8, 4 spp; *Z*10, 3 spp; *Z*12, 2 spp). Only *Myrmicaria eumenoides* appears to have exploited the full potential of alkenes, producing nine different positional isomers and ten different positional dienes. These may be used in nest-mate recognition because it has been shown that the ant *Formica exsecta* uses nest-specific distributions of $C_{21:1}$ to $C_{33:1}$ *Z*9-alkenes for nest-mate discrimination (Martin et al. 2008a). The true alkene diversity will be

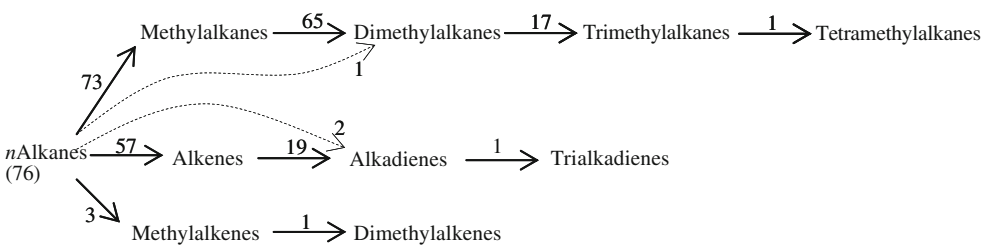

Fig. 1 Number of species (over arrows) producing cuticular hydrocarbons (CHCs) belonging to each of the ten CHS subgroups

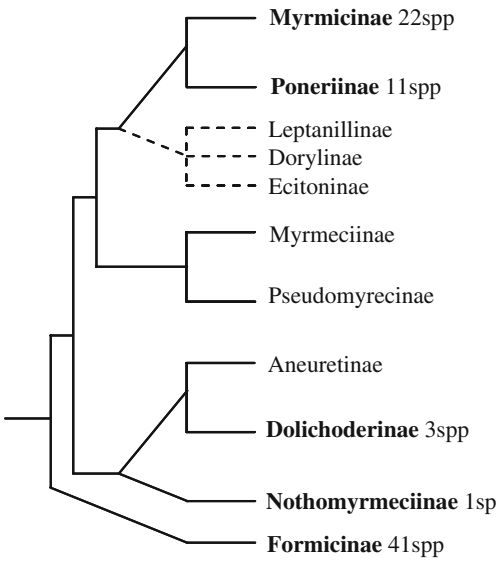

Fig. 2 Subfamily phylogeny within the Formicidae adapted from Hölldobler and Wilson (1990)

revealed only with the systematic determination of double bond positions.

Currently only three species (Table 1) produce CHCs that contain both double bonds and methyl branches (Fig. 1). Interestingly, one of these species is *Nothomyrmecia macrops,* the most primitive known ant, which produces large amounts (65%) of methylalkenes and methylalkadienes. This manifestation is biochemically complex since it requires pathways that combine both the insertion of double bonds and methyl groups. This suggests that the genetic architecture for the production of all hydrocarbons was already present in ancestral ants, and has been maintained throughout their subsequent vast radiation. This parallels the finding that the key biosynthetic gene subfamily used in pheromone production existed prior to the radiation of Lepidoptera (Liénard et al. 2008). Although few species currently appear to synthesize this type of complex CHC profiles, a recent study that used high temperature (HT) columns to analyze CHC profiles of two species of tropical ants (Menzel et al. 2008) found that *Crematogaster modiglianii* and *Camponotus rufifemur* produce 14 methylalkenes, all of which occurred at chain-lengths greater than C_{34}. In fact, in these two species, more than 96% of the total ion chromatogram (peak area) is composed of CHCs with a chain-length greater than C_{34}. Chain lengths this long can only be reliably detected by using HT columns.

In summary, the vast majority of compounds occurred at chain-lengths between C_{19} and C_{33}, with $C_{25}/C_{27}/C_{29}$

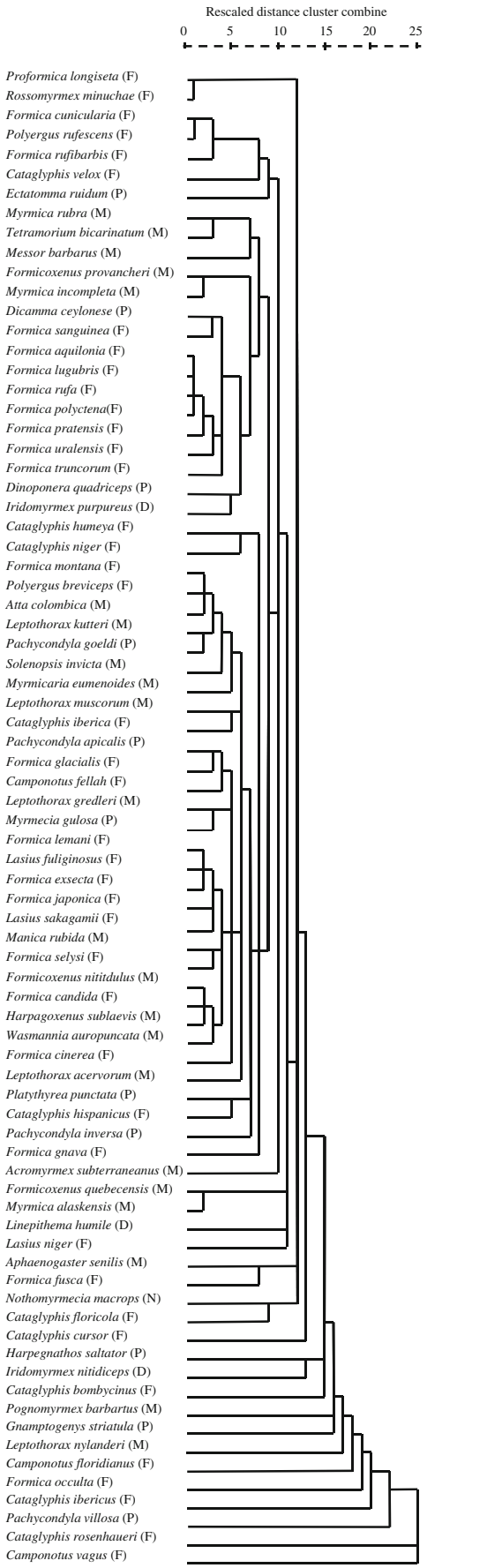

Fig. 3 Dendrogram of ant species (Hymenoptera: Formicida derived from a hierarchical cluster analysis of published cuticular hydrocarbon data. Subfamilies are Myrmicinae (M), Poneriinae (P), Dolichoderinae (D), Nothomyrmeciinae (N), and Formicinae (F)

compounds usually being the most abundant. However, this seems to be a reflection of the limitations of the GC columns used rather than a true reflection of the ants' biosynthetic capabilities. For example, *Formica tuncorum* and *F. pratensis* have been analyzed using both standard (Nielsen et al. 1999; Martin et al. 2008c) and HT columns (Akino 2006; Martin and Drijfhout 2009a). In these species, CHCs larger than C_{34} accounted for 56% (*F. tuncorum*) and 51% (*F. pratensis*) of the total ion chromatogram (peak area) of the profile. Current HT columns can reach 440°C, and can detect chain-lengths up to C_{60}. Alternatively, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has been used to detect CHCs up to C_{60} in termites (Cvačka et al. 2006).

The 78 ant species included in this review were grouped into their respective five subfamilies (Fig. 2) based on the phylogeny given in Hölldobler and Wilson (1990).

The cluster tree based on all the CHCs (Fig. 3) and the phylogenetic tree (Fig. 2) showed no similarity, with species from several subfamilies producing the same CHCs (Table 1). For example, species belonging to three different subfamilies (*Dinoponera quadriceps*, *Iridomyrmex purpureus*, and several *Fromica* spp. [Fig. 2]) are clustered together (Fig. 3) because they all produced some of the same CHCs such as 11,15- and 13,17-dimethyls, several monomethyls, and *n*-alkanes (Table 1). Even very closely related species (e.g., *Formica fusca* and *Formica lemani*) can be very different chemically, with *F. fusca* workers producing dimethyls but no alkenes whereas *F. lemani* workers produce only *Z*9-alkenes and *n*-alkanes (Martin et al. 2008b). Cvačka et al. (2006) also noticed that neither GC-MS nor MALDI-MS were able to describe the phylogenetic relationships among the 12 species of diverse insect taxa studied (termites, ants, a cockroach, and a flesh fly).

The image of an ant as a chemical factory is well supported by this review, with the 78 species producing about 1,000 individual hydrocarbons. Using the basic *n*-alkane backbone, ants have exploited two major biochemical pathways, the introduction of double bonds and of methyl branches. This has allowed them to produce the rich and varied CHC profiles that we see. In the future, the use of HT columns and a systematic determination of bond and methyl group positions will add significantly to the already remarkable hydrocarbon diversity found in ants. It appears that complex CHCs were already biosynthesized by primitive ants, and this may explain why no association between CHC profiles and phylogeny was found in either this or previous studies (Morgan et al. 2003; Cvačka et al. 2006). The ubiquity of *n*-alkanes and many monomethlyalkanes means that they are unlikely to convey complex information among ants, whereas the diversity of unsaturated compounds and especially the dimethyls make them excellent discriminatory compounds. This is further supported by the finding that almost all ants so far studied produce either alkenes or dimethyls, but rarely both. Furthermore, ants control the chain lengths of these key groups of CHCs in order to encode species (Martin et al. 2009c) and nest-mate information (Martin et al. 2008a). The ability of ants to produce such a large diversity of CHCs, having a range of biophysical properties, has helped them adapt to environments that range from tropical to arctic, as well as to develop a complex chemical communication system.

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When a male chrysomelid encounters a conspecific female, it first touches the female with its antennae and palpi, then mounts her, and finally mates. Both physical surface stimuli or chemical contact stimuli of the females might elicit this male mating behavior. Upon disturbance and contact, adult leaf beetles of the subfamily Chrysomelinae are known to release defensive secretions from elytral and pronotal glands (Deroe and Pasteels 1982). Defensive secretions of species of the subtribe Chrysomelina, including the genus *Phaedon,* contain isoxazolinone glycosides that act as deterrents against ants (Pasteels et al. 1982; Sugeno and Matsuda 2002). Thus, when a male encounters a conspecific adult, it may be exposed to both highly polar compounds (from defensive glands) and to non-polar CHC.

We investigated the mating behavior of the mustard leaf beetle *Phaedon cochleariae* (F.) (Chrysomelidae: Chrysomelinae) and studied the role of cuticular chemical contact stimuli for mating induction. First, we recorded parameters of mating behavior of males with living conspecific males and females; the percentage of mating attempts and duration of matings with living conspecifics were used as reference for further bioassays with crude cuticular extracts of male and female *P. cochleariae.* Active extracts were subjected to fractionation and further bioassays to characterize the compounds that elicit mating behavior. In addition, we analyzed the CHC profiles of male and female beetles by GC-MS and compared the compositions by multivariate statistics.

Methods and Materials

Beetles All *P. cochleariae* used for bioassays and chemical analyses were taken from a laboratory colony maintained in a climate chamber at 20°C, 70% relative humidity, and a 16:8 hL:D cycle. Newly emerged beetles were separated by sex; batches of 50 beetles of the same sex were kept together in plastic containers (20×20×6.5 cm; Gerda, Schwelm, Germany) containing moist paper towels and food. All life stages were reared on leaves of Chinese cabbage, *Brassica rapa* L. ssp. *pekinensis*.

Crude Dichloromethane Extracts of Beetles Prior to extraction, 21-d-old beetles were killed by freezing at −18ºC for 30 min and thawed for 15 min at room temperature. For the initial bioassays, crude extracts from males and females were prepared by extracting batches of 42 beetles of each sex for 10 min in 1 ml dichloromethane. For fractionation, another extract was prepared by extracting 250 females for 10 min in 3 ml dichloromethane. These extracts were concentrated under a gentle stream of nitrogen to dryness, dissolved in 420 µl and 2.5 ml dichloromethane, respectively [i.e., 1 beetle equivalent (BE) per 10 µl], and were stored at −18°C until used for bioassays, chemical analysis, or fractionation.

Fractionation of Extracts A dichloromethane extract prepared as described above was subjected to fractionation according to the scheme outlined in Fig. 1. After each fractionation step, the fractions were concentrated to dryness and dissolved in dichloromethane so that the concentration of each fraction always corresponded to 1 BE per 10 µl. First, the extract was fractionated by silica gel column chromatography (Pasteur pipettes filled with 600 mg silica gel 60; particle size 0.063–0.200 mm, Merck, Darmstadt, Germany). The column was pre-washed with 1 ml hexane; the samples were loaded; and compounds were eluted with 1.4 ml hexane (the CHC) and 1.4 ml methanol (defensive compounds). Both fractions were bioassayed. The methanol fraction also was used for thin layer chromatography. The hexane fraction was further fractionated by $AgNO_3$ column chromatography (Pasteur pipettes filled with 350 mg 10% $AgNO_3$ in silica gel;≥ 99%, Fluka, Buchs, Switzerland) to separate saturated hydrocarbons from olefins. The column was pre-washed with 1 ml hexane; the samples were loaded; and the

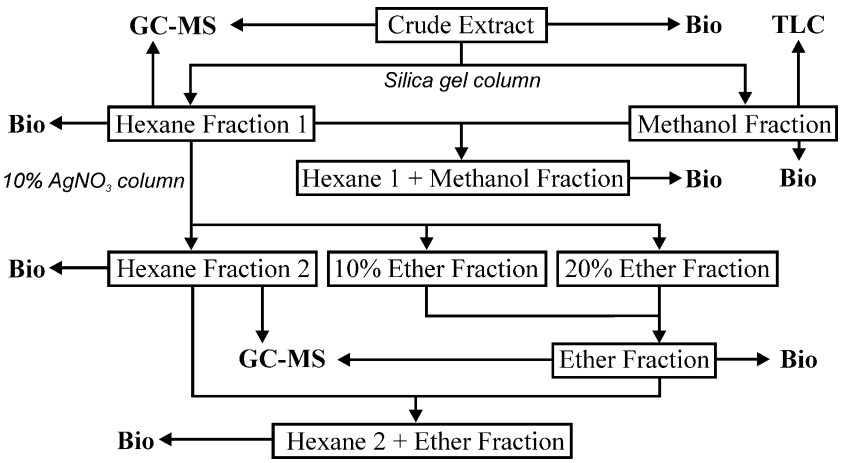

Fig. 1 Fractionation scheme for the cuticular crude dichloromethane extract of 250 female *Phaedon cochleariae*. "Bio" means that 400 µl (40 beetle equivalents=BE) of these fractions were used for (40) behavioral bioassays. GC-MS and TLC refer to extracts that where analyzed by GC-MS (2 BE) and TLC (80 BE), respectively

hydrocarbons were eluted successively with 1.4 ml each of hexane, 10% and 20% butyl methyl ether in hexane, respectively. Saturated hydrocarbons eluted in the hexane fraction and olefins in the combined ether fraction. Both fractions were tested separately and in combination in bioassays. All fractions were stored at −18°C until used in bioassays or chemical analysis.

Behavioral Bioassays All bioassays were conducted in Petri dishes (5.5×1.2 cm) lined with filter paper between 13:00 and 15:00 h CET at 22–24°C. A Petri dish was illuminated by a 60 W bulb placed 40 cm above the dish; no daylight or other light was available in the bioassay room. To guarantee sexual maturity only 21-d-old beetles were used. Test males were kept individually in Petri dishes for at least 1 d before the bioassay was begun to obviate male-male mating attempts just prior to the tests. A bioassay was initiated by placing a test male in a dish. We recorded whether a test male started a copulation attempt, and if so, how long this attempt lasted. A bioassay was terminated after 10 min, unless the test male still attempted to mate. In this case, the trial was terminated at the end of the attempt. Thus, we determined "% male response", i.e., the percentage of males exhibiting a copulation attempt, and "duration of copulation", i.e., the time a male took for copulation. For each bioassay, N=40 males were tested, and each one was offered a new mating object.

In a first step, we analyzed the mating behavior of males towards conspecific, living males and females. A single potential mate was placed in a Petri dish 10 min prior to bioassay start. In a second step, we offered a dead male or female to a test male. The potential mates were killed by freezing (1 h at −18°C), and the dead specimens were glued onto the filter paper in the middle of a Petri dish 24 h before the beginning of the trials (5Star™ glue stick; Code: 296026). This set-up excluded the possibility that behavior of the partner influences male mating behavior. In a third step, we investigated the role of chemical cues for male mate choice. Bioassays were performed with cuticular extracts and fractions that were applied to glass beads (KnorrPrandell, Lichtenfels, Germany, manufacturer information: 4×3 mm; dark green Rocailles-drop). A single glass bead was glued onto the filter paper in the center of a Petri dish and was treated with 10 µl of the extract or fraction to be tested (1 BE) 24 h before the trial started to allow evaporation of the solvent. Crude dichloromethane extracts of both sexes were tested, whereas all other fractions used for bioassays were from females only.

Chemical Analysis Chemical identification and quantification of CHC were performed on a coupled gas chromatograph-mass spectrometer system (7890A GC – 5975C MSD; Agilent, Waldbronn; Germany), equipped with a cold-injection system (CIS4, Gerstel, Mühlheim a. d. Ruhr, Germany). A 1 µl aliquot of each sample was injected at 150°C, and the CIS was immediately heated to 300°C at 12°C/s. A fused silica column (DB-5MS, 30 m×0.32 mm i.d.×0.25 µm film thickness, J & W Scientific, Folsom, CA, USA) was used for separation with a constant helium flow of 1.2 ml/min. The oven temperature program was started at 100°C and then heated to 300°C at a rate of 5°C/min (50 min). Electron impact ionization was 70 eV.

Hydrocarbons were identified by their mass spectra (Nelson and Sukkestad 1970; Nelson et al. 1972; Pomonis et al. 1980) and corroborated by their retention indices (Kováts 1965; Carlson et al. 1998). The double-bond positions of trienes were characterized by their typical mass fragments (Karunen 1974; Conner et al. 1980; Millar 2000). The double-bond positions of monoenes and dienes were determined by interpreting the mass spectra of the dimethyl disulfide (DMDS) derivatives (Francis and Veland 1981). We used the 10% ether fraction containing monoenes and the 20% ether fraction with dienes (Fig. 1) for DMDS derivatization. Methyl-branched alkenes were hydrated under a H_2 and D_2 atmosphere, respectively, by using palladium on activated charcoal as a catalyst to determine the position of the methyl group and the relative position of the methyl group to the double bound position (see electronic supplementary material; Fig. 1). Peak areas relative to total peak areas were calculated. Peaks with areas <0.1% were not included in the quantitative analyses.

For quantification of CHC, 10 µl of an *n*-eicosane solution (0.1 mg/ml in hexane) were added as an internal standard (IS) to 20 µl of each crude extract and fraction, respectively. Each of these samples contained 2 BE. For quantification of CHC and subsequent discriminant analysis, individual beetles (N=40 for each sex) were extracted for 10 min each in 125 µl dichloromethane, and 10 µl IS was added for quantification.

Thin layer chromatography (TLC) of the methanol fractions (80 BE) was conducted in order to determine whether the methanol fraction contained defensive compounds. TLC was performed by using silica gel 60 F_{254} plates (0.2 mm, 20×20 cm, Merck, Darmstadt, Germany) developed with chloroform:methanol in a ratio of 8:2 (*v*:*v*) (Sugeno and Matsuda 2002). After development, spots were recorded under UV_{254}-light and visualized with thymol-sulfuric acid (Stahl 1967).

Statistical Analysis All statistical analyses were conducted with SPSS 17 (SPSS Inc., Chicago, IL, USA) or Statistica 7.0 (StatSoft Inc., Tulsa, OK, USA). Differences in the percentage of males responding to the potential mates in each bioassay were analyzed by χ^2-tests, and differences in copulation duration were analyzed by the Kruskal-Wallis H test. Data were corrected for multiple comparisons by using the Bonferroni-Holm-correction.

A canonical discriminant analysis (DA) was performed to determine whether CHC profiles of males and females could be separated based on the relative composition of compounds. Each peak area was transformed according to the Aitchison's formula (Aitchison 1986):

$$Z_{ip} = 1\mathrm{n}\left[\frac{A_{ip}}{g(A_p)}\right],$$

where A_{ip} is the area of peak i for beetle p; $g(A_p)$ is the geometric mean of all peaks for beetle p; and Z_{ip} is the transformed area of peak i for beetle p. To apply the transformation formula to profiles that did not possess all peaks, the constant 0.01 was added to each relative peak area (Aitchison 1986; Steiger et al. 2007). Variables for the DA were selected based on U-values calculated by Mann-Whitney U test statistics (measurement for between-group differences). Low U-values indicate high inter-group variability. Two separate DAs were performed based on the 25 peaks with the lowest U-values, either selected from all hydrocarbons or from saturated hydrocarbons only (active fraction in bioassays). Wilks' λ and the percentage of individuals correctly classified by a leave-one-out cross validation were used to assess the quality of the DAs.

Results

Chemistry of Cuticular Extracts The CHC profiles of male and female *P. cochleariae* contain the same compounds; 67 peaks were detectable in both sexes (Table 1, Fig. 2). Hydrocarbons of males and females included *n*-alkanes, monomethyl alkanes, dimethyl alkanes, olefins with one, two, or three double bonds, and monomethyl branched alkenes. The chain-length of the hydrocarbons ranged from C19 to C45. The profiles of males and females were dominated by long-chained methyl-branched alkenes (42.1±7.0% and 52.0±4.6%, of total peak area in male and female CHC profiles, respectively) and 2-methylalkanes with even-chained carbon backbones (31.7±9.7% and 23.0±7.2%, respectively), whereas *n*-alkanes represented only a small amount of the total hydrocarbons (1.0±0.3% and 0.7±0.2%, respectively). The most prominent peaks were 2-MeC28, 27-MeC39-16/18-ene, 29-MeC41-18/20-ene, and 31-MeC43-20/22-ene. The mass spectra of the DMDS derivates of unbranched monoenes showed the typical fragments for double bonds at position 7 (*m/z* 145/M-145) or 9 (*m/z* 173/M-173), and those of 6,9-dienes were characterized by fragments at *m/z* 131, *m/z* 155, and *m/z* 203. Trienes showed distinct fragments at *m/z* 108, $[CH_3CH_2(CH{=}CH)_3H]^+$ and M-56, $[H(CH{=}CH)_3R]^+$, characteristic for 3,6,9-trienes (Millar 2000). Methyl-branched alkenes had varying double bond positions, and those of them with chain lengths ≥C31 had a constant spacing of 7 or 9 methylene-groups between the methyl-branching and the double bond. Counting from the ω-end of the molecules, we detected 13-MeC$_x$-21-ene and 15-MeC$_x$-23-ene (7 CH_2-groups), or 12-MeC$_x$-22-ene, 13-MeC$_x$-23-ene, 14-MeC$_x$-24-ene, and 15-MeC$_x$-25-ene (9 CH_2-groups).

Although the CHC profiles of males and females were qualitatively similar, the profiles were sex specific with respect to relative CHC quantities. Significantly different patterns of relative CHC compositions were detected both when running a DA based on 25 peaks either selected from all 67 hydrocarbons or when conducting the DA based on 25 peaks selected from saturated hydrocarbons only (marked with c and d, respectively, in Table 1). The canonical roots of both DAs clearly separated the male from female CHC pattern (Fig. 3; all hydrocarbons: Wilks' $\lambda=0.119$, $\chi^2=139.2$, $P<0.001$; saturated hydrocarbons: Wilks' $\lambda=0.135$, $\chi^2=130.4$, $P<0.001$). In the DA based on all hydrocarbons, 95.0% of the cross-validated cases were correctly classified according to their sex. In the DA based on saturated hydrocarbons, 91.3% were correctly classified by cross-validation.

The TLC analysis revealed that surface washes of *P. cochleariae* with dichloromethane not only extracted CHC but also defensive compounds. Three major spots that correspond to literature R_f-values for nitropropanoyl isoxazolinone glycosides typical for adults of the subtribe Chrysomelina (Sugeno and Matsuda 2002) were detected in the methanol fraction (*Rf* 0.27, 0.43, and 0.54, respectively).

Behavioral Bioassays The majority of the test males attempted to mate with living females (88%) and males (93%) (Fig. 4A), but they copulated significantly longer with females (Fig. 4B). Similar results were obtained in bioassays in which dead males or females were offered to test males (Fig. 4). When crude dichloromethane extracts of males or females were applied to glass beads, the percentage of males attempting to mate with the beads treated with male or female crude extract was as high as with living or dead beetles (Fig. 4A). The duration of the mating attempts with the glass beads treated with male crude extract was as long as with beads with female crude extract (Fig. 4B). Altogether, the percentage of the test males showing mating attempts did not differ among all the treatments, but there were significant differences in the duration of the copulation attempts.

The non-polar hexane fraction 1 (Fig. 2C) elicited mating in the test males, but the percentage of males attempting to mate was significantly lower than to that elicited by the crude extract. The polar methanol fraction containing the defensive compounds did not elicit any copulation responses (Fig. 5A). The male responses to the combination of hexane

Table 1 Relative quantities (mean % total peak area ± s.d.) of cuticular hydrocarbons of male and female *Phaedon cochleariae* (*N*=40)

No[a]	RI[b]	Compound	Males	Females
1	1943	7-MeC19	0.21±0.23	0.13±0.10
2[c]	2269	6,9-C23diene	3.85±1.84	1.78±0.95
3[c]	2274	9-C23ene	2.25±1.16	1.14±0.39
4[d]	2336	11/13-MeC23	0.12±0.06	0.09±0.03
5[c,d]	2385	5,13-diMeC23	0.16±0.10	0.05±0.04
6[c,d]	2462	2-MeC24	1.77±0.90	3.19±1.07
7	2479	3,6,9-C25triene	0.14±0.27	0.94±0.58
8	2482	7-C25ene	0.12±0.07	0.23±0.13
9[c,d]	2500	*n*-C25	0.09±0.07	0.19±0.09
10	2507	13-MeC25-7-ene / 15-MeC25-7-ene	0.35±0.20	0.32±0.15
11[c,d]	2534	11-/13- MeC25	0.52±0.24	1.25±0.46
12[c,d]	2563	2-MeC25	0.10±0.05	0.16±0.05
13[d]	2573	7,13-diMeC25	0.52±0.22	0.44±0.15
14[d]	2582	5,13-diMeC25	0.15±0.07	0.10±0.05
15	2609	14-MeC26-3-ene / 14-MeC26-8-ene / 15-MeC26-9-ene	0.24±0.12	0.19±0.09
16[b]	2633	11-/12-/13-MeC26	0.09±0.06	0.13±0.07
17	2664	2-MeC26	4.03±1.36	4.41±0.97
18	2676	6,9-C27diene	0.23±0.15	0.35±0.17
19	2676	9-C27ene	0.14±0.20	0.20±0.11
20[c]	2682	3,6,9-C27triene	0.43±0.50	1.58±1.15
21	2682	7-C27ene	0.21±0.15	0.10±0.04
22	2707	13-MeC27-2-ene / 15-MeC27-4-ene / 15-MeC27-9-ene	2.15±1.05	1.76±0.63
23[d]	2732	13-MeC27	1.14±0.47	1.66±0.70
24[c,d]	2764	2-MeC27 / 9,13-/9,15-diMeC27	0.61±0.12	0.50±0.09
25	2782	5,13-/5,15-diMeC27	1.19±0.54	1.30±0.33
26[c,d]	2800	*n*-C28	0.53±0.27	0.25±0.12
27[c,d]	2862	2-MeC28	20.85±5.00	12.45±3.50
28	2876	6,9-C29diene	0.47±0.47	0.15±0.21
29	2878	9-C29ene	0.38±0.37	0.10±0.17
30[c]	2882	3,6,9-C29triene	0.20±0.14	0.05±0.09
31[d]	2893	4,10-/4,12-/4,14-diMeC28	0.06±0.08	0.11±0.07
32[d]	2900	*n*-C29	0.37±0.16	0.28±0.09
33	2931	13- /15-MeC29	1.07±0.47	1.13±0.40
34	2958	11,15-diMeC29	0.10±0.06	0.08±0.05
35[c,d]	2963	2-MeC29	0.30±0.08	0.15±0.14
36	2982	5,13-/5,15-diMeC29	0.09±0.05	0.10±0.05
37	3005	3,13-/3,15-diMeC29	0.16±0.06	0.17±0.05
38	3031	12-/13-/14-MeC30	0.15±0.60	0.05±0.05
39[d]	3063	2-MeC30	5.03±2.45	2.97±1.66
40[c]	3084	9-C31ene	0.12±0.13	0.02±0.07
41	3102	2,12- / 2,14-diMeC30	0.15±0.27	0.14±0.20
42	3109	17-MeC31-6/8-ene/ 19-MeC31-8/10-ene [e]	0.35±0.24	0.38±0.26
43[d]	3132	13-MeC31	1.23±0.46	1.09±0.35
44	3205	3,13-/3,15-diMeC31	0.58±0.27	0.48±0.14
45	3281	5,13-diMeC32	0.09±0.08	0.10±0.12
46[c,d]	3331	13-MeC33	0.84±0.25	0.64±0.19
47[d]	3382	5,13-diMeC33	1.46±0.47	1.23±0.30
48[d]	3405	3,13-diMeC33	0.31±0.13	0.26±0.13
49[c]	3506	21-MeC35-10/12-ene / 23-MeC35-12/14-ene	5.97±2.22	2.98±1.18

Table 1 (continued)

No[a]	RI[b]	Compound	Males	Females
50	3532	13-/15-MeC35	0.71±0.28	0.76±0.17
51[d]	3572	7,13-/7,15-diMeC35	0.41±0.23	0.29±0.13
52	3582	5,13-/5,15-diMeC35	1.22±0.32	1.16±0.25
53	3607	23-MeC36-12-ene / 24-MeC36-13-ene / 25-MeC36-14-ene	0.54±0.35	0.39±0.35
54[d]	3605	3,13-/3,15-diMeC35	0.47±0.35	0.31±0.32
55[c,d]	3631	12-/13-/14-/15-/16-MeC36	0.06±0.06	0.19±0.12
56	3708	23-MeC37-12/14-ene / 25-MeC37-14/16-ene	6.79±1.86	5.50±1.06
57[c,d]	3731	13-/15-MeC37	0.21±0.13	0.38±0.16
58	3772	7,13-/7,15-diMeC37	0.11±0.09	0.10±0.08
59[c]	3808	25-MeC38-14-ene / 26-MeC38-15-ene / 27-MeC38-16-ene	0.21±0.22	0.54±0.35
60	3804	3,13-/3,15-diMeC37	0.20±0.17	0.20±0.15
61[c]	3909	25-MeC39-14/16-ene / 27-MeC39-16/18-ene	6.25±3.29	14.03±4.43
62[c]	4008	27-MeC40-16-ene / 28-MeC40-17-ene / 29-MeC40-18-ene	0.22±0.19	0.59±0.27
63[c]	–[f]	27-MeC41-16/18-ene / 29-MeC41-18/20-ene	4.92±1.56	9.42±1.30
64[c]	–	29-MeC42-18-ene / 30-MeC42-19-ene / 31-MeC42-20-ene	0.55±0.22	0.89±0.28
65	–	29-MeC43-18/20-ene / 31-MeC43-20/22-ene	11.77±3.45	12.88±3.47
66	–	31-MeC44-20-ene / 32-MeC44-21-ene / 33-MeC44-22-ene	0.23±0.19	0.46±0.26
67	–	31-MeC45-20/22-ene / 33-MeC45-22/24-ene	1.58±0.58	1.71±0.61

[a] No=Peak numbers referring to Fig. 2.

[b] RI=Retention index.

[c] Peak was selected for DA based on all hydrocarbons.

[d] Peak was selected for DA based on saturated hydrocarbons.

[e] 17-MeC31-6/8-ene refers to 17-MeC31-6-ene+17-MeC31-8-ene.

[f] No reference alkanes were available for the calculation of the retention indices.

and methanol fraction did not differ significantly from those to the crude dichloromethane extract or to the hexane fraction (Fig. 5A). The duration of copulations did not differ when the glass beads were treated with dichloromethane extract, hexane fraction, or with a combination of hexane and methanol fraction. Alkanes (hexane fraction 2; Fig. 2D) elicited as much mating behavior as the total CHC fraction (hexane fraction 1; Fig. 2C), whereas alkenes (ether fraction; Fig. 2E) elicited no mating behavior at all (Fig. 5A). The percentage of males showing mating attempts towards the combination of alkane and alkene fractions was the same as to the alkanes alone (Fig. 5A), but the duration of mating attempts differed when comparing these types of potential mating objects (Fig. 5B). Test males tried to mate with beads treated with the combined alkane and alkene fractions longer than with beads treated only with the alkane fraction.

Discussion

Cuticular hydrocarbons (CHC) play an essential role in male mating behavior of the mustard leaf beetle, *P. cochleariae*. The CHC profile of this species is a complex mixture of straight-chained and methyl-branched saturated and unsaturated hydrocarbons with a wide range of carbon backbones (C19 – C45). The high percentage of 2-methyl alkanes seems to be typical for chrysomelids, representing major compounds in the CHC profiles of nearly all leaf beetle species investigated (Dubis et al. 1987; Nikolova et al. 1999; Nelson et al. 2002, 2003; Nelson and Charlet 2003; Peterson et al. 2007; Geiselhardt et al. 2009). The large quantities of methyl-branched alkenes in *P. cochleariae* is striking. This substance class has been reported from only a few insect species but across a wide taxonomic range, including Lepidoptera (Francke et al. 2000), Coleoptera (Golden et al. 1992), Diptera (Sonnet et al. 1979; Etges and Jackson 2001), and Hymenoptera (Brown et al. 1990; Menzel et al. 2008). The same methyl-branched alkenes as detected in *P. cochleariae* have been reported from the tropical ant *Camponotus rufifemur* (Menzel et al. 2008), where remarkably, this substance class comprised up to 98% of all hydrocarbons. The function of these compounds in *P. cochleariae* remains unclear since they are not involved in male mate recognition, as test males did not respond to the alkene fraction. The only single fraction that elicited male mating behavior was the saturated hydrocarbon fraction. This fraction consisted mainly of 2-methyl alkanes (60.2%), other

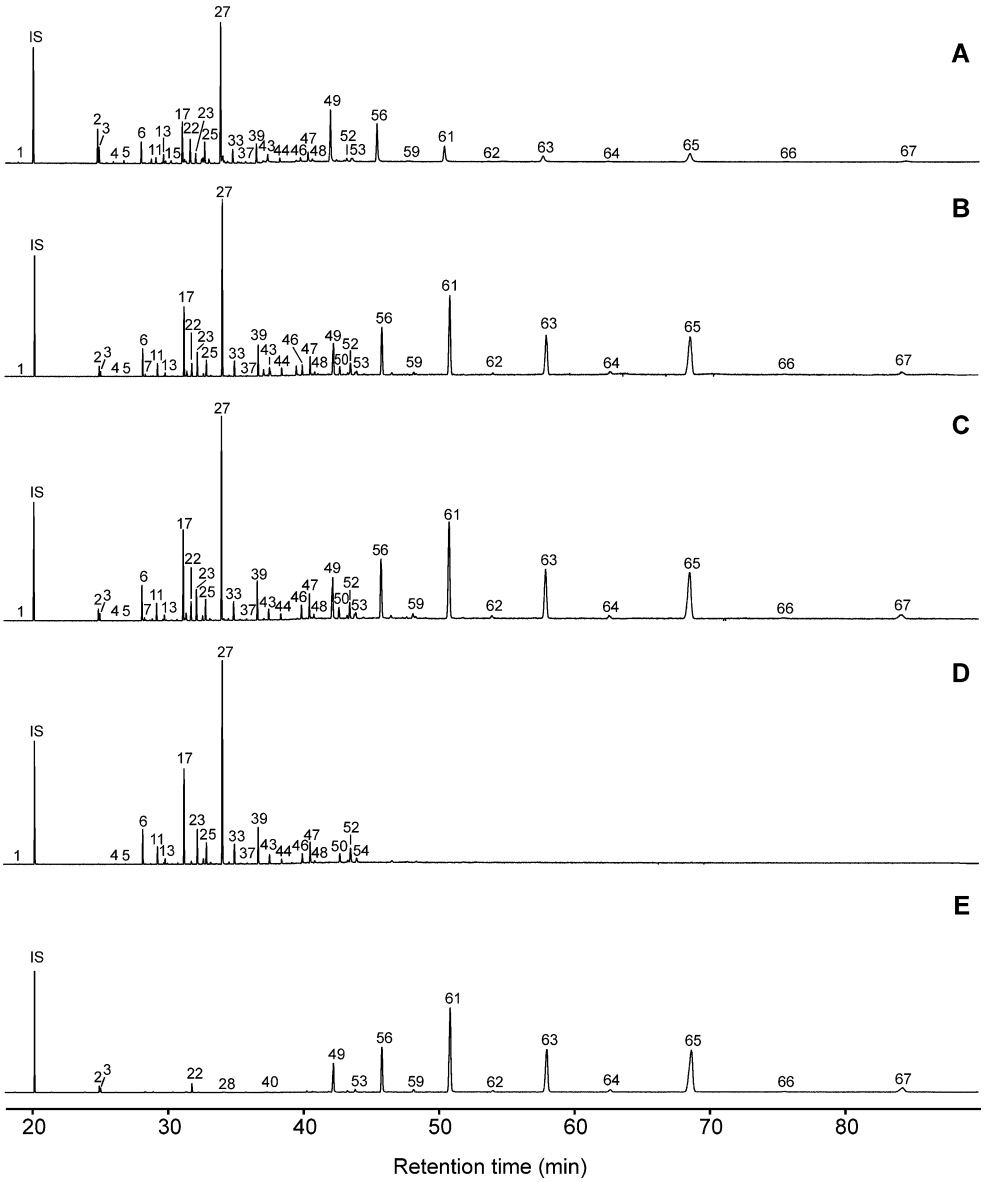

Fig. 2 Total ion current chromatograms of the cuticular crude dichloromethane extracts from (**A**) male or (**B**) female *Phaedon cochleariae*, and of (**C**) the total CHC fraction (hexane fraction 1), (**D**) the saturated hydrocarbons (hexane fraction 2), and (**E**) the unsaturated hydrocarbons (ether fraction) from females. Designation of fractions corresponds to Fig. 1. Numbers refer to numbers in Table 1. IS=internal standard

monomethyl alkanes (20.4%), dimethyl alkanes (17.7%), and only a small proportion of *n*-alkanes (0.9%). The only chrysomelid in which the active compounds of the contact pheromone have been characterized is *G. atrocyanea* (Sugeno et al. 2006). Males of this species respond mainly to 9-MeC27, 11-MeC27, 9-MeC29, and 11-MeC29, whereas 13-MeC27, 13-MeC29, 15-MeC29, and *n*-alkanes elicit only a low activity.

The CHC profiles of *P. cochleariae* showed no sex-specificity in their qualitative composition. The qualitative CHC profiles of males and females were also similar in other leaf beetle species such as the Colorado potato beetle, *L. decemlineata* (Dubis et al. 1987; Nelson et al. 2003), the northern corn rootworms, *Diabrotica barberi* and *D. longicornis*, (Golden et al. 1992), and the blue milkweed beetle, *C. cobaltinus*, (Peterson et al. 2007). Nevertheless, the relative proportions of the compounds differed between the sexes. In *C. cobalticus*, these differences were perceived by males and used for sex recognition (Peterson et al. 2007). Male *C. cobalticus* attempted to copulate with dummies treated with cuticular extract from conspecific females but not with dummies treated with male extract. In contrast, *P. cochleariae* males did not discriminate between males and females, even though DAs of CHC profiles of males and females clearly demonstrated that theses could be separated based on CHC quantities. Dummies treated with male cuticular extract elicited as much mating response as dummies treated with female extracts. Thus, CHC in *P. cochleariae* are used as a general copulation-eliciting pheromone rather than as a sex pheromone.

Males of the two-spotted ladybird beetle, *Adalia bipunctata*, also lack the ability to discriminate between sexes by using CHC (Hemptinne et al. 1998). In this species, sex recognition is based on the behavior of the potential mates.

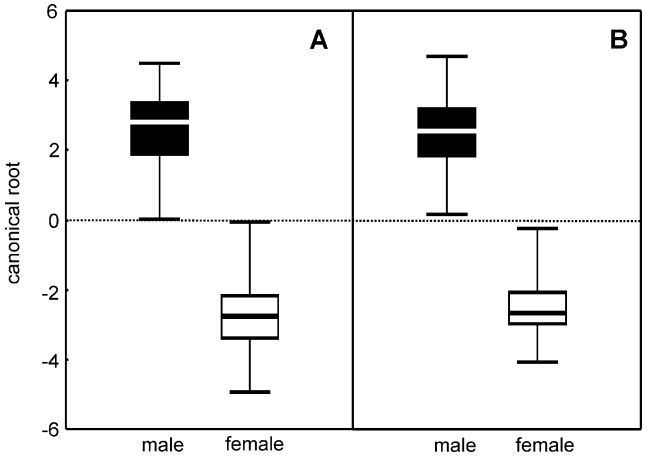

Fig. 3 Canonical roots of the discriminant analysis of cuticular hydrocarbon (CHC) profiles of *Phaedon cochleariae* males and females (40 for each sex) based on the 25 CHC with the lowest *U*-values for between-group differences in a Mann-Whitney *U* test. Peak selection was based on (**A**) all hydrocarbons or (**B**) on saturated hydrocarbons only (active fraction in bioassays). Boxes indicate 25% and 75% percentiles, bars inside boxes are medians, and whiskers indicate minima and maxima

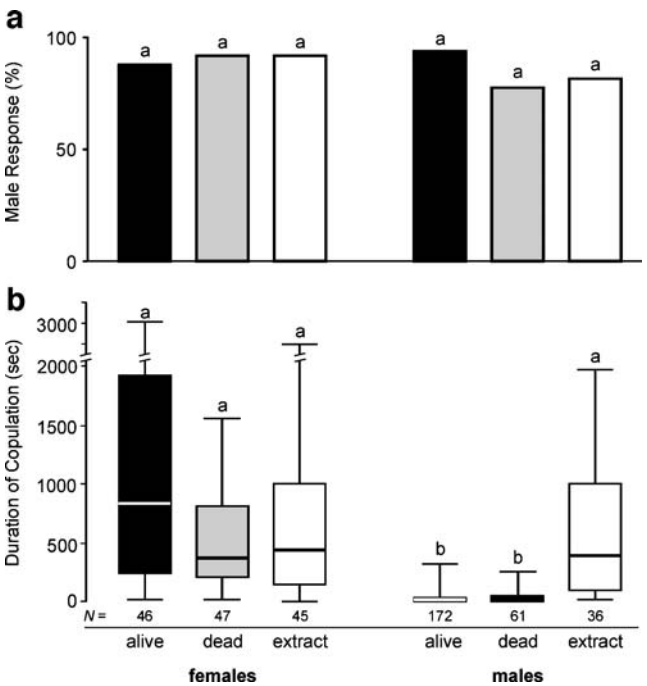

Fig. 4 *Phaedon cochleariae* male mating responses (**A**) and duration of copulation attempts (**B**) with conspecific living or dead females and males, respectively, and with glass dummies treated with cuticular crude extracts from females or males. Mating response test: N=40 (= 100%) for each trial. Boxes indicate the 25% and 75% percentiles, the bars inside the boxes are the medians, and whiskers indicate the minima and maxima. Different letters above columns and whiskers indicate significant differences evaluated by a χ^2-test (**A**) or a Kruskal-Wallis H test (**B**) (α=0.05 for both tests). All data were Bonferroni-Holm-corrected for multiple comparisons

Females encountered by a male stop moving and thus signal their readiness to mate, whereas encountered males try to run away and escape. In *P. cochleariae*, sex recognition seems to be mediated by physical contact cues rather than sex-specific behavioral cues since test males were able to discriminate between dead males and females. This discrimination ability was not detectable when considering percentage of mating attempts, but when recording copulation duration. Males copulated much longer with dead females than with dead males. Similar results were obtained with living beetles. Interestingly, the test males attempted to mate with glass dummies treated with crude extracts of males or females as long as with dead or living females. Thus, copulation duration differed when males encountered male and female bodies, but was the same when males encountered glass beads treated with male or female extracts. The short duration of copulation with males might be due to physical stimuli of male bodies that reduce the time of mating attempt. This implies that males recognize a

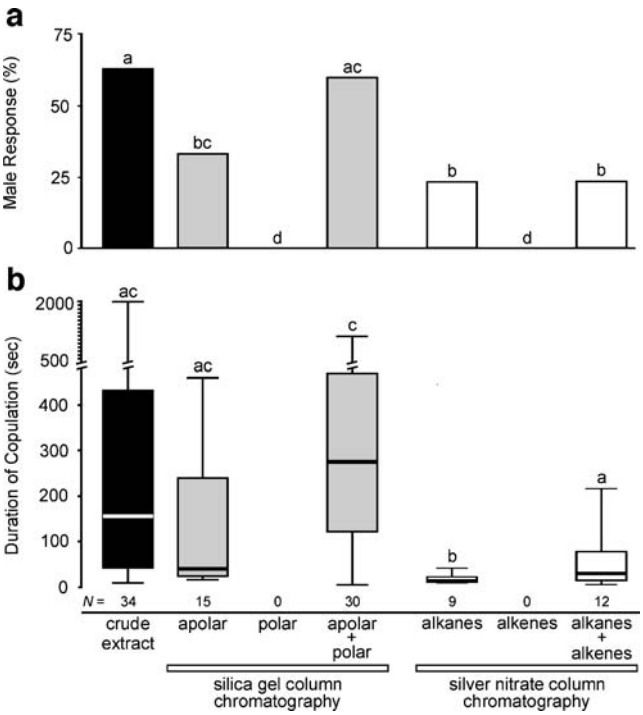

Fig. 5 *Phaedon cochleariae* male mating responses (**A**) and duration of copulation attempts (**B**) with glass dummies treated with either cuticular crude extracts of conspecifics (*black*) or fractions of this extract obtained by silica gel column chromatography [grey: hexane (*non-polar*), methanol (*polar*), and combination of both fractions (non-polar+polar)] or fractions obtained by 10% silver nitrate column chromatography [white: hexane (*alkanes*), ether (*alkenes*), and combination of both fractions (*alkanes*+ *alkenes*)]. Mating response test: N=40 (= 100%) for each treatment. Boxes indicate the 25% and 75% percentiles, the bars inside the boxes are the medians, and whiskers indicate the minima and maxima. Different letters above columns and whiskers indicate significant differences evaluated by means of χ^2-test (**A**) or Kruskal-Wallis H test (**B**) (α=0.05 for both tests). All data were Bonferroni-Holm-corrected for multiple comparisons

conspecific adult by CHC that elicit mating behavior, but discrimination between male and female adults is due to tactile cues. As this tactile sex recognition mechanism works rapidly in *P. cochleariae*, selection pressure on males to discriminate the sexes by CHC may be weak.

Although the defensive compounds released from the elytral and pronotal glands did not induce mating behavior in males when tested alone, we cannot rule out the possibility of defensive compounds functioning synergistically to the CHC in eliciting copulation behavior. Albeit not significant after the Bonferroni-correction, the combination of CHC and extract containing defensive secretion tended to elicit mating attempts more frequently than CHC alone, and males copulate longer with dummies treated with the mixture of both fractions. In the rove beetle, *Aleochara curtula*, defensive compounds act synergistically with female cuticular sex pheromones (Peschke 1983), but in this species small quantities of defensive secretion alone elicit male copulation attempts. Further studies are required to clarify the potential role of the defensive compounds in the mating behavior in *P. cochleariae*.

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have been based on colony-specific cuticular hydrocarbons as putative recognition cues. Furthermore, Nunes et al. (2008) demonstrated differences in the chemical profiles between workers of *Frieseomelitta varia* and *Lestrimelitta limao*, which might explain the aggressive behavior observed between these species. When, however, conspecific individuals from a different colony but with similar chemical profile were introduced into a colony of *F. varia*, no aggression towards the "intruder" was observed (Nunes et al. 2008). Pianaro et al. (2007) investigated the chemical hydrocarbon profile of individuals from mixed colonies of *Melipona rufiventris* and *M. scutellaris*, and also determined the chemical composition of the colony's cerumen (i.e., beeswax mixed with plant resins). They suggested that there might be a form of "chemical competition" between the species that inhabit the same mixed colony.

Given that there are more than 400 species of stingless bees (Michener 2000), the chemical composition and role of cuticular hydrocarbons in this bee group have remained poorly investigated, especially when compared with other social insect groups. Therefore, the present study aimed to answer whether there are differences in the cuticular hydrocarbon profiles among castes, ages, and sexes of the stingless bee, *F. varia*.

Methods and Materials

Insects

The study was performed with four colonies of *F. varia*, each housed in glass-covered wooden boxes (35×24×7 cm). The colonies were kept in the laboratory and were connected to the outside through a plastic tube, which allowed free foraging of the bees.

Bees were collected individually in Eppendorf tubes (1.5 ml) and killed by freezing prior to extraction. We used 20 old workers, identified by their dark-colored scutelum, and 20 newly emerged workers, identified by their white coloration and by their activities inside the colony. In addition to the workers, 7 newly emerged males, collected inside the colony, and 8 old males, collected in a male cloud in front of a colony, as well as 6 virgin queens and 9 physogastric queens (i.e., queens with high ovarian activity) were included in the study. Old and newly emerged workers were taken from all four experimental colonies, whereas newly emerged males were collected from two different colonies. Since we captured the old males in a male cloud, their colony-origin could not be identified. The virgin queens from two different colonies were collected about 4 days after emergence. From each experimental colony, we analyzed one physogastric queen. Four physogastric queens were used for the analysis of the overall chemical composition of the cuticular hydrocarbons and 5 to identify the position of double bonds through derivatization with dimethyl-disulfide (DMDS). Due to the small sample size, however, the queens were not considered in the statistical analysis.

Hydrocarbon Extraction and Analysis

Prior to the extraction, the workers' last pair of legs was cut off to avoid contamination with resins, since old workers of *F. varia* usually carry large amounts of this material. Cuticular hydrocarbons were extracted by immersing the insects in hexane for 1 min. Samples were analyzed by gas chromatography-mass spectrometry (GC-MS), and compounds were identified through a comparison of the mass spectra from the extracts with those in the Wiley library and data of diagnostic ions present in the literature. Substances found at relative concentrations below 5% and those present in fewer than three analyzed individuals in the same experimental group were represented as traces in the table of relative concentrations (Table 1). For the quantification of the substances, we calculated the area under the respective peak in the chromatogram. Analyses were carried out by using a Shimadzu QP2010 GC-MS equipped with a DB-5MS (J&W Scientific, Folsom, CA, USA) capillary column (30 m×0.25 mm×0.25 µm). The GC injection port was set to 250°C and the transfer line to 280°C. The column temperature was held at 150°C increasing to 280°C at 3°C/min, held at 280°C for 15 min, and then raised to 300°C at 5°C/min, and then held for 15 min. Helium was used as carrier gas at 1 ml/min, and samples were injected in the splitless mode. Electron impact mass spectra were measured at 70 eV with a source temperature of 250°C. Identification of the double bond positions of alkenes and alkadienes was made through derivatization of a crude hexane extract from five physogastric queens with DMDS (Carlson et al. 1989). The extract was dried with nitrogen and re-suspended in 200 µl of magnetically stirred hexane. Subsequently, 200 µl of DMDS (Sigma-Aldrich, São Paulo, SP, Brazil) and 100 µl of iodine solution (dissolved in diethyl ether, 6% p/v) were added. The vial was then purged with nitrogen, closed, and agitated at ambient temperature for 24 h. Thereafter, the mixture was diluted in hexane and 5% sodium thiosulfate solution, thereby extracting the organic phase, which was subsequently dried with sodium sulfate and analyzed by GC-MS.

Statistical Analyses

The chemical profiles of the different groups were compared by multivariate statistical analysis. Principal component analysis (PCA) was used to define the main peaks for the comparison. Stepwise discriminant analysis was applied to contrast the individuals of different sex and

Table 1 Relative concentrations of compounds identified from hexane extracts of the cuticle of workers, males, and queens of *Frieseomelitta varia*

Ret time[a]	Compound	DI[a]	Young workers		Old workers		Young Males		Old Males		Virgin Queens		Phy queens[a]	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
12.62	Pentadecanol	*Wiley Library*	–		0.57±0.56		0.46±0.27		–		*Traces*		*Traces*	
14.73	Palmitic acid	*Wiley Library*	–		2.36±2.79		tr		–		*Traces*		*Traces*	
15.73	Palmitic Acid Ethyl Ester	*Wiley Library*	tr[b]		2.12±2.86		0.55±0.40		0.20±0.11		0.55±0.05		*Traces*	
17.44	11 - Octadecene-1ol	*Wiley Library*	tr		1.71±2.36		0.33±0.24		tr		0.39±0.20		*Traces*	
18.17	3 - Octadecenal	*Wiley Library*	–		0.55±0.65		tr		–		–		*Traces*	
18.64	Heneicosane (C21)	296	0.86±0.44		1.10±2.08		3.00±3.06		tr		0.71±0.47		*Traces*	
19.38	Linoleic Acid	*Wiley Library*	–		0.54±0.46		tr		tr		–		*Traces*	
20.27	Linoleic Acid Ethyl Ester	*Wiley Library*	–		2.70±2.87		0.15±0.07		tr		0.52±0.83		–	
20.46	Oleic Acid Ethyl Ester	*Wiley Library*	–		5.83±5.83		tr		tr		0.66±0.56		3.49±5.87	
21.05	N-Butyl Palmitate	*Wiley Library*	–		tr		tr		0.29±0.28		3.13±2.10		0.31±0.32	
21.50	Olealdehyde	*Wiley Library*	–		2.41±3.57		0.34±0.29		0.67±0.62		*Traces*		*Traces*	
23.22	Z - Eicosyne	*Wiley Library*	–		2.17±2.23		*Traces*		–		–		*Traces*	
23.35	9-Tricosene	322 (416, 173, 243)[c]	0.53±0.40		0.33±0.19		0.50±0.33		*Traces*		0.17±0.22		–	
24.13	Tricosane (C23)	324	5.40±3.66		2.05±3.09		8.41±7.18		0.35±0.26		0.44±0.31		0.21±0.21	
26.77	Tetracosane (C24)	338	0.57±0.24		0.41±0.54		1.50±1.01		0.08±0.03		–		0.12±0.05	
28.45	9 - Pentacosene	350 (444, 173, 271)	tr		tr		–		–		–		0.21±0.13	
28.57	8 - Pentacosene	350 (444, 159, 285)	tr		0.17±0.06		0.13±0.09		*Traces*		*Traces*		0.30±0.22	
28.68	7 - Pentacosene	350 (444, 145, 299)	tr		0.11±0.01		0.13±0.05		*Traces*		*Traces*		0.20±0.16	
28.75	5 - Pentacosene	350 (444, 117, 327)	0.26±0.03		tr		0.22±0.29		*Traces*		–		1.65±2.94	
29.48	Pentacosane (C25)	352	37.15±9.26		2.51±2.11		24.50±10.46		6.13±3.93		6.81±3.12		11.82±14.70	
32.01	Hexacosane (C26)	366	1.18±0.12		0.54±0.27		2.47±2.86		1.05±0.26		0.38±0.12		0.43±0.06	
33.43	10 - Heptacosene	378 (472, 187, 285)	–		–		–		–		–		1.14±0.45	
33.50	13 - Heptacosene	378 (472, 229, 243)	0.61±0.66		1.65±2.33		0.55±0.29		*Traces*		0.18±0.05		1.88±1.01	
33.78	7 - Heptacosene	378 (472, 145, 327)	0.61±0.43		0.62±0.95		0.81±0.51		0.36±0.38		0.23±0.14		1.18±0.75	
33.94	5 - Heptacosene	378 (472, 117, 355)	4.70±8.96		tr		0.53±0.80		0.07±0.03		*Traces*		1.72±0.22	
34.45	Heptacosane (C27)	380	34.19±9.44		26.84±15.05		24.83±14.75		49.76±11.36		16.98±5.64		11.31±2.91	
35.20	9 and 11 and 13 - Methylheptacosane	395, 141, 280, 169, 252, 197, 224	0.82±0.58		0.33±0.22		0.93±0.68		*Traces*		–		–	
35.60	5 - Methylheptacosane	394, 85, 336	*Traces*		0.30±0.18		0.09±0.04		*Traces*		–		*Traces*	
36.16	3 - Methylheptacosane	394, 43, 378	–		0.97±0.73		0.25±0.22		*Traces*		*Traces*		*Traces*	
36.80	Octacosane (C28)	394	0.28±0.15		0.58±0.44		3.87±4.66		0.93±1.85		0.14±0.05		0.31±0.29	
37.56	9,17 - Nonacosadiene	*Table 2*	–		*Traces*		*Traces*		0.18±0.07		–		0.54±0.44	
37.76	7,17 - Nonacosadiene	*Table 2*	–		–		0.16±0.13		*Traces*		–		0.65±0.44	
38.27	12 - Nonacosene	406 (500, 215, 285)	0.38±0.28		–		–		1.26±2.11		*Traces*		3.99±2.61	

Table 1 (continued)

Ret time[a]	Compound	DI[a]	Young workers		Old workers		Young Males		Old Males		Virgin Queens		Phy queens[a]	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
38.40	7 - Nonacosene	406 (500, 145, 355)	0.57±0.31		0.42±0.45		0.88±0.62		0.31±0.38		0.44±0.46		2.84±1.52	
38.60	5 - Nonacosene	406 (500, 117, 383)	–		0.67±0.16		*Traces*		*Traces*		*Traces*		3.93±0.74	
39.10	Nonacosane (C29)	408	10.27±2.87		10.05±4.90		5.45±4.71		19.57±6.40		5.70±1.36		5.63±2.39	
39.76	11 and 13 and 15 - Methylnonacosane	422, 169, 280, 197, 252, 225, 224	0.99±0.79		0.77±0.57		0.42±0.21		0.24±0.06		–		*Traces*	
40.18	3, 11 - Dimethylnonacosane	436, 57, 183, 155, 281	0.59±0.29		0.85±0.47		0.14±0.11		*Traces*		*Traces*		0.08±0.07	
40.72	3 - Methylnonacosane	422, 43, 406	*Traces*		2.61±1.69		*Traces*		*Traces*		*Traces*		0.09±0.03	
41.20	Triacontane (C30)	422	0.60±0.72		0.29±0.17		2.16±2.10		0.15±0.05		*Traces*		*Traces*	
42.05	9,17 - Hentriacontadiene	*Table 2*	–		–		–		*Traces*		*Traces*		1.31±0.66	
42.23	9,19 - Hentriacontadiene	*Table 2*	*Traces*		*Traces*		–		*Traces*		–		2.04±0.71	
42.40	8,22 - Hentriacontadiene	*Table 2*	–		*Traces*		–		*Traces*		–		2.82±0.65	
42.55	14 - Hentriacontene	434 (528, 243, 285)	*Traces*		–		–		*Traces*		–		4.61±1.12	
42.80	9 - Hentriacontene	434 (528, 173, 355)	–		–		*Traces*		*Traces*		*Traces*		3.44±2.81	
43.00	7 - Hentriacontene	434 (528, 145, 383)	–		–		–		*Traces*		–		2.00±0.69	
43.46	Hentriacontane (C31)	436	2.71±1.22		0.82±0.53		0.80±0.80		2.32±1.45		1.18±0.71		2.03±0.83	
44.08	11 and 13 - Methylhentriacontane	450, 169, 308, 197, 280	1.41±0.99		1.02±1.59		0.45±0.19		0.23±0.23		–		–	

[a] Ret, retention; DI, Diagnostic ions; Phy, physogastric

[b] tr, trace amount

[c] Diagnostic ions in ( ) are from the mass spectra of DMDS derivatives

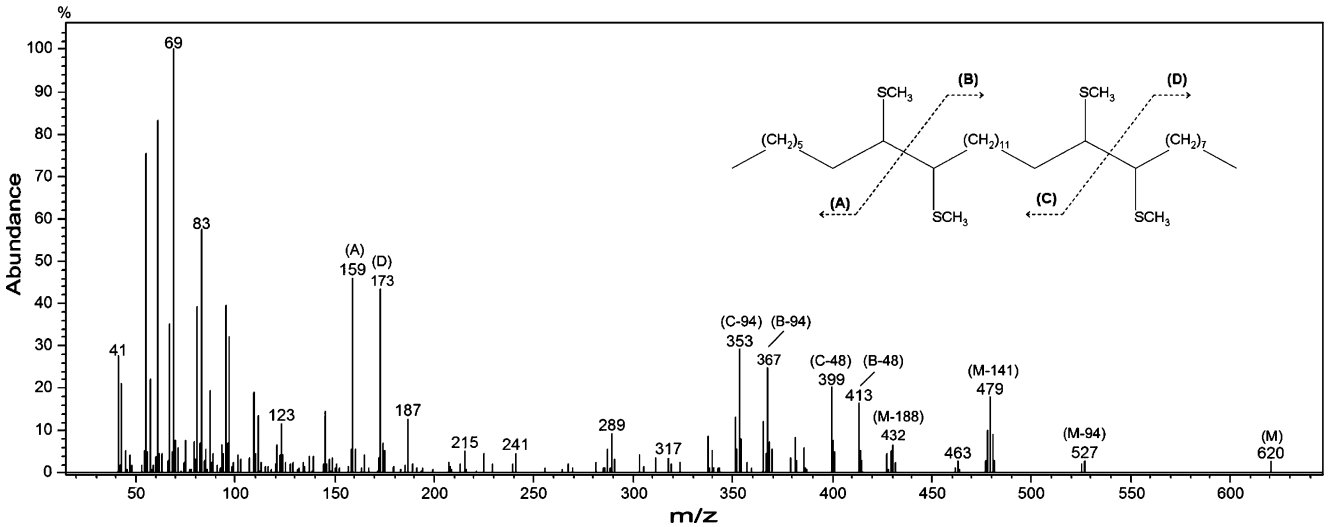

Fig. 1 Mass spectrum of DMDS derivative of 8,22–hentriacontadiene from a cuticular extract of five physogastric queens of *Frieseomelitta varia*

ages. Compounds missing in most individuals of an analyzed group as well as compounds contributing less than 5% to the first two factors, as indicated by the PCA, were excluded from the statistical analysis. The relative concentrations of the compounds used for the discriminant analysis were readjusted to 100%. To avoid errors in compositional sample data, the area under each peak was transformed according to the following formula: $Z = \ln [A_p / g (A_p)]$, where A_p is the area under the peak, $g (A_p)$ is the geometric mean for each individual compound group and Z is the transformed peak area. (Aitchison, 1986)

Results

The analysis of the chemical compounds of the cuticle of *F. varia* revealed a total of 48 substances. The majority of these compounds were hydrocarbons, although other classes of substances such as alcohols and acids were present as well. The identified hydrocarbons included saturated and unsaturated alkanes, alkenes, alkynes, and alkadienes. The compounds varied from 21 to 31 carbon atoms in chain length. The most abundant substances in all analyzed groups of these bees were alkanes (Table 1). Among these, the most abundant was heptacosane for all groups except for the physogastric queens, in which the relative proportion of pentacosane was higher (Table 1). Diagnostic ions of DMDS derivatives were used to identify the double bond positions of the alkadienes (Fig. 1, Table 2). Although different isomers co-eluted, the identifications were based on the most abundant isomer in the peak.

Young workers showed a much lower chemical diversity than old workers. In addition, we found differences in the concentration of alkanes between these two bee groups. Here, alkanes with 23 and 25 carbon atoms were present in higher relative amounts in young workers than in old workers. Furthermore, half of the branched compounds identified in old workers were not found or found only as traces in recently emerged workers.

The comparison among males of different ages demonstrated that younger individuals presented a higher diversity

Table 2 Diagnostic ions from the major isomers of DMDS-derivatized alkadienes from hexane-extracted cuticle of *Frieseomelitta varia*

Compound	Diagnostic ion (m/z)[a]									
	$(A)^+$	$(D)^+$	$(B\text{-}48)^+$	$(B\text{-}94)^+$	$(C\text{-}48)^+$	$(C\text{-}94)^+$	$(M - 188)^+$	$(M - 141)^+$	$(M - 94)^+$	$(M)^+$
9,17 - Nonacosadiene	173	215	371	325	329	283	404	451	498	592
7,17 - Nonacosadiene	145	215	399	353	329	283	404	451	498	592
8,22 - Hentriacontadiene	159	173	413	367	399	353	432	479	526	620
9,17 - Hentriacontadiene	173	243	399	353	329	283	432	479	526	620
9,19 - Hentriacontadiene	173	215	399	353	357	311	432	479	526	620

[a] $(M)^+$ = molecular ion. Fragments A-D deduced as described in Carlson et al. (1989)

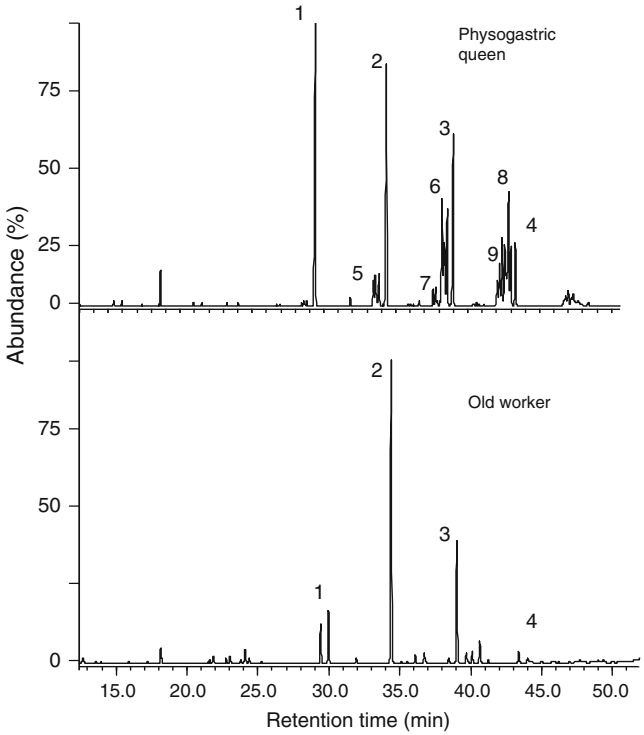

Fig. 2 Comparison between the cuticular composition of an old worker and a physogastric queen of *Frieseomelitta varia* showing differences in identified compounds between these groups. 1) C_{25}; 2) C_{27}; 3) C_{29}; 4) C_{31}; 5) *Z*-C_{27}*; 6) *Z*-C_{29}*; 7) *ZZ*-C_{29}*; 8) *Z*-C_{31}*; and 9) *ZZ*-C_{31}. (*) indicates the presence of multiple isomers

of cuticular compounds than did older individuals. As was the case with workers, young males showed higher relative amounts of tricosane and pentacosane, whereas old males showed higher relative amounts of heptacosane, nonacosane, and hentriacontane.

Comparing workers and males of the same age, we found that the cuticular extract from young workers showed a lower compound richness than that of recently emerged males. The opposite was true for the extract from old individuals, in which higher substance diversity was observed in workers. Regarding the presence or absence of specific compounds, young males show a chemical cuticular profile closer to that of old workers.

Cuticular extracts from virgin queens did not contain the major compounds identified from the other groups. In these queens, we also found higher proportions of *n*-butylpalmitate, which was either absent, or present in low concentrations, in almost all other bee groups investigated.

Physogastric queens showed a particular cuticular hydrocarbon profile. In this group, we found only traces of compounds with lower molecular weight, whereas hydrocarbons with double bonds were present in high relative amounts. Alkenes and alkadienes with 27, 29, and 31 carbon atoms occurred in much higher relative quantities in the extracts from physogastric queens than in extracts from the other analyzed groups. Alkenes and alkadienes with 31 carbon atoms were found almost exclusively in the physogastric queens. These compounds were present only as traces, if present at all, in the other groups. On the other hand, alkanes present in high relative amounts in the cuticle of the other groups (C27 and 29) were far less abundant in the physogastric queens. An examination of the chromatograms of cuticular extracts from physogastric queens shows that, as the chain length increases in carbon number, alkanes occur in lower relative amounts, whereas alkenes and alkadienes occur in higher relative amounts (Fig. 2).

Factor 1 of the principal component analysis (PCA) described 39.86% of the observed variation. Factor 1 + factor 2 described 61.30% of the total variation, whereas the sum of the first three factors described 73.10% of the observed variation. The stepwise discriminant analysis of the subset of identified compounds significantly separated the analyzed bee groups (Global model: Wilks's λ = 0.0046, $F_{15,36}$ = 14.55, P<0.001). The compounds responsible for the separation were pentacosane, hexacosane, heptacosane, 7-nonacosene, and hentriacontane. In addition, the canonical analysis demonstrated that all groups were significantly separated from each other (Fig. 3). The classification matrix indicated that a total of 81% of the individuals were allocated correctly to their predicted group; if allocation were random, the expected correct allocation would be 25%. All comparisons of treatment groups showed significant differences in the discrminant analysis (Table 3).

Discussion

The chemical analysis of the cuticular compounds of *F. varia* showed clear differences between workers and males

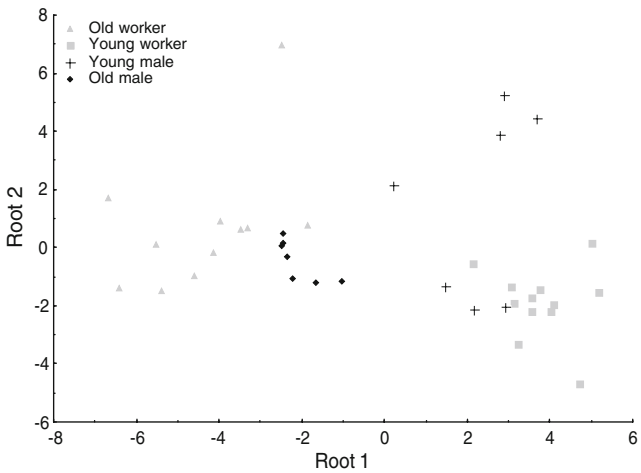

Fig. 3 Discrimination between *Frieseomelitta varia* males and workers of different ages based on cuticular hydrocarbon phenotypes

Table 3 Discriminant analysis of cuticular compounds from males and workers of *Frieseomelitta varia* of different ages

Combinations			Mahalanobis Distance	$F_{5,13}$	P
young workers	*vs*	old workers	83.36	32.45	< 0.001
young workers	*vs*	young male	33.86	13.18	< 0.001
young workers	*vs*	old male	35.62	17.60	< 0.001
old workers	*vs*	young male	78.13	23.90	< 0.001
old workers	*vs*	old male	19.84	7.28	< 0.01
young male	*vs*	old male	41.85	15.36	< 0.001

and between two age classes within these groups. For example, extracts from young individuals contained higher relative amounts of alkanes with shorter chains, such as tricosane and pentacosane, compared to older individuals, suggesting that these compounds might be a chemical cue that indicates the age of individual *F. varia* bees. Queens demonstrated a distinct chemical profile from workers and males. Yet, due to the small number of individuals analyzed, the cuticular profile of the queens could not be compared statistically to those of the workers or the males. However, our results are in accordance with many studies of cuticular compounds of social insects, which suggest that these substances could be used as chemical signals to carry information about caste, age, sex, and reproductive status of the individuals (reviewed in Howard and Blomquist 2005).

Many of the compounds identified in the cuticlular extracts of old workers of *F. varia* also have been described from other species of stingless bees (e.g., *Melipona bicolor*: Abdalla et al. 2003; *M. scutellaris* and *M. rufiventris*: Pianaro et al. 2007; *Scaptotrigona bipunctata*: Jungnickel et al. 2004; *F. varia* and *Lestrimelitta limao*: Nunes et al. 2008; *Schwarziana quadripunctata*: Nunes et al. 2009). The main compounds in these species have been demonstrated to be hydrocarbons with chain lengths varying from 19 to 33 carbon atoms, of which odd chain compounds occurred in higher relative amounts. With the exception of *M. bicolor*, cuticular extracts of these species contained high relative amounts of alkanes, mainly those with 27 and 29 carbon atoms. In *M. bicolor*, cuticular extracts from most classes of individuals contained alkenes as the major components. In accordance with our findings for *F. varia*, both quantitative and qualitative differences between gender and age have been described for *M. bicolor* (Abdalla et al. 2003). In contrast to *F. varia*, however, young workers of *M. bicolor* did not differ from virgin queens in cuticular hydrocarbon profile. For *S. quadripunctata*, Nunes et al. (2009) recently described differences among young workers, old workers, and virgin queens. However, the study did not analyze cuticular hydrocarbons from males and physogastric queens.

Since colony influence on individual odor constitution and its role in nest-mate recognition have already been described in detail elsewhere for *F. varia* (Nunes et al. 2008), in the present study we focused attention on aspects of cuticle chemical profile information not related to nest defense, and we did not consider colony as a variable in the anlaysis. The source of chemical compounds that mediate recognition behaviors, and the importance of each component in the cuticular hydrocarbon complement in this species will be addressed elsewhere.

In young workers, we found a lower diversity in the chemical composition of their cuticle than in old workers. These findings are similar to what is known from honeybees, in which both the diversity of the cuticular substances and their concentrations are lower in young than in old individuals (Breed et al. 2004). These differences between the age groups could be related to the time of exposure to potential odor sources (Breed et al. 1995, 2004; Downs and Ratnieks 1999).

Several studies on both social and solitary insects have indicated qualitative differences in the chemical composition of the cuticle, or differences in the relative concentration of the compounds, in relation to the individuals' gender. Yet, some studies found the same chemical compounds with the same relative concentrations in both sexes (Howard and Blomquist 2005). Here, we found both qualitative and quantitative differences in the chemical composition of the cuticle between males and workers. Although little is known about this subject in social insects, behavioral tests with hexane extracts or purified compounds have demonstrated that other insects such as the longhorned beetles use chemical differences on the cuticular surface for gender recognition (reviewed in Allison et al. 2004).

Recent studies with the European beewolf showed that individuals from the same family have more chemical similarities than individuals from different families (Herzner et al. 2006). The authors suggest that females can assess these chemical similarities and avoid mating with related males. A similar type of investigation with *F. varia* would provide an interesting context for the data set presented here. However, the method used to collect old males (i.e., in a male cloud) impeded us from knowing their colony origins, so we would not be able to make comparisons between the family status and the chemical composition of the males.

In *F. varia*, the cuticle of young males presented a richer chemical profile than that of old males. The observed absence of certain compounds in old males could be due to the fact that these individuals leave the hive within a few days after their emergence. Consequently, as demonstrated in honeybees, the lack of contact with colony-internal odor sources could lead to a reduced diversity in cuticular compounds (Breed et al. 1995, 2004; Downs and Ratnieks 1999).

In stingless bees, with the exception of *Melipona*, virgin queens exhibit a conspicuous behavior immediately after their emergence (Imperatriz-Fonseca and Zucchi 1995). Often, these unfertilized gynes move around rapidly inside the colony and are frequently assessed by workers. In the Meliponini, the three possible fates of virgin queens are (a) to substitute for the physogastric queen; (b) to get killed by the workers; or (c) to participate in a swarming event (Sakagami 1982). It is still unknown, how the workers identify a virgin queen immediately after her emergence. The characteristic morphology and behavior of these gynes could act as signals for their recognition by workers, and the chemical compounds associated with their cuticle could serve as an additional source of information. Thus, workers would be able to distinguish virgin queens from other individuals by either the presence or absence of certain chemical compounds, or by differences in compound concentrations. Moreover, in *F. varia*, the virgin queens might be identifiable due to the relatively high proportions of *n*-butylpalmitate present on their cuticle.

In many groups of social insects, the mechanism by which queens retain their reproductive status and recognize the status of other individuals is still unknown. Although in the honeybees, pheromones from the mandibular glands have been shown to fulfill these functions, many recent studies indicate that in other social insect species cuticular hydrocarbons are the primary cues of fertility (Monnin et al. 1998; Peeters et al. 1999; Howard and Blomquist 2005). According to Keller and Nonacs (1993), the mandibular queen pheromone in honeybees does not have the function of a control pheromone, but serves as a signal to indicate the queen's presence in the nest and to stimulate behaviors of workers that raise their fitness and, indirectly, that of the queen. Our results, based on other pheromones described, support the idea that the cuticular hydrocarbons in *F. varia* might be responsible for indicating the queen's presence in a colony. Endler et al. (2004) demonstrated that the queens of the ant *Camponotus floridanus* "mark" their eggs with cuticular hydrocarbons, and that these compounds indeed act as indicators for the queen's presence resulting in non-laying workers. The compounds identified in the cuticle of *F. varia* queens certainly do not result in worker sterility, since the workers are sterile even in the absence of the queen (Boleli et al. 1999). Yet, similar to *C. floridanus*, the queen's cuticular hydrocarbons could indicate her presence in the colony, thereby initiating all the vital worker-queen interactions such as cohort, feeding, and other behaviors related to the provisioning and oviposition process.

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(e.g., *Chrysoperla*) subsist on only nectar and pollen (Brooks and Barnard 1990).

Green lacewing adults have long been known to communicate intraspecifically via substrate vibrations (e.g., Henry 1982), but only recently was it discovered that males in the genus *Chrysopa* produce aggregation pheromones from thousands of elliptically shaped glands embedded in their abdominal cuticle (Chauhan et al. 2004; Zhang et al. 2004, 2006a, b). Interestingly, female goldeneyed lacewings [Neuroptera: Chrysopidae: *Chrysopa* (= *Co.*) *oculata* Say] do not enter traps baited with pheromone (1*R*,2*S*,5*R*,8*R*-iridodial) (Chauhan et al. 2004; Zhang et al. 2004), presumably because females attract males via substrate-borne vibrations at close range (Henry 1982). However, Chauhan et al. (2007) showed that significant numbers of wild *Co. oculata* females are attracted to the vicinity of iridodial dispensers in the field, and observed that the attracted females laid eggs on soybean leaves near the dispensers. Thus, the judicious application of *Chrysopa* pheromones may provide a practical means to conserve and augment these lacewings in the field for biological control of pests.

All adult chrysopids possess paired prothoracic glands (PG) thought to produce defensive secretions (allomones) (Güsten and Dettner 1991; Szentkirályi 2001). In the course of ongoing research efforts to find aggregation pheromones for other chrysopid species, we have investigated or reinvestigated the PG secretions of ten species from five genera in the tribe Chrysopini. Blum et al. (1973) identified skatole (3-methylindole) as the compound responsible for the stench of adult goldeneyed lacewings (*Co. oculata*), with the major non-odorous (to humans) component of the secretion (93%) reportedly being 1-tridecene. The presence of skatole in PG secretions of *Chrysopa* spp. was verified by Güsten and Dettner (1991), but these authors found *(Z)*-4-tridecene in species representing five genera of Chrysopini, including six *Chrysopa* species. However, Güsten and Dettner (1991) did not include spectral data or chemical details as to how the double bond position and geometry of tridecene were determined. More recently, Zhu et al. (2000) identified *(Z)*-4-tridecene as the sole major constituent of the PG secretion in *Cl. carnea*, but they also described the secretion as "offensive-smelling", which is enigmatic since skatole was not present and tridecenes are not strong or foul smelling.

Here, we report on analyses of PG secretions for the following green lacewings from North and South America, Australia, and China: *Ceraeochrysa* (= *Ce.*) *cubana* (Hagen) (Brazil); *Chrysopa* (= *Co.*) *oculata* Say, *Co. nigricornis* Burmeister, *Co. incompleta* Banks, *Co. quadripunctata* Burmeister (USA), and *Co. septempunctata* Wesmael (China); *Chrysoperla* (= *Cl.*) *rufilabris* (Burmeister) (USA) and *Cl.* sp. (Brazil); *Plesiochrysa ramburi* (Schneider) and *Mallada* spp. (Australia).

Methods and Materials

Insects and Preparation of Extracts The sources, method of sampling the adult insects, identity, and the specialists responsible for identifying the lacewings are listed in Table 1. Identifications were aided by reference to Penny et al. (2000) and Brooks and Barnard (1990). Traps used to catch live chrysopids were modeled after a previously described trap (Aldrich et al. 1984). Iridodial lures were prepared as described before (Chauhan et al. 2007), except that octane was used as solvent instead of heptane. The plant volatile lure was prepared in an analogous manner using β-caryophyllene/ 2-phenylethanol/ methyl salicylate (1:1:1 by volume; total active ingredient=5 mg). Chemical extracts were prepared in ca. 50 µl of methylene chloride (EMD Chemicals Inc., Gibbstown, NJ, USA) by either extracting the whole thorax (earlier samples) or by excising the glands from the prothorax (later samples). The paired prothoracic glands were easily removed by fastening a lacewing, dorsal side up, under water in a dissecting dish, transversely cutting the dorsal intersegmental membrane between the prothorax and mesothorax, and making a dorso-medial incision in the prothorax exposing the bluish-colored glands in each side of the prothorax. Each gland, attached to a small piece of cuticle, was then removed with fine forceps, gently dried with tissue paper, and extracted in about 20 µl of solvent (CH_2Cl_2 or hexane) for analysis.

Chemical Standards The following commercially available standards were used: 1-tridecene, hexanoic acid, octanoic acid, skatole, 3-hexanol, 2-hexanol, tridecanol, benzaldehye, 2-phenylethanol, methyl salicylate (Aldrich Chemical Co., Milwaukee, WI, USA); nonanoic acid (Emery Industries, Cincinnati, OH, USA); nonanal, decanal, and β-caryophyllene (Bedoukian Research, Danbury, CT, USA). N-3-Methylbutylacetamide and N-3-methylbutylpropanamide were prepared by reactions of 3-methylbutylamine with acetyl and propanyl chlorides, respectively, at 0°C in the presence of triethylamine (Heath and Landolt 1988). *(Z,Z)*-4,7-Tridecadiene was synthesized starting with a −78°C suspension of hexyltriphenylphosphonium bromide (1.04 g, 2.44 mmol) in 20 ml of THF to which a 1M THF solution of lithium bis (trimethylsilyl)amide (2.3 ml, 2.3 mmol) was added. The reaction mixture was warmed to −30°C over 30 min, kept at this temperature for 1 h, and then cooled to −78°C. To this clear orange ylide solution, a 0.5 ml THF solution of *(Z)*-3-hepten-1-al [112 mg, 1 mmol; prepared by Swern oxidation (Chauhan et al. 1994) of *(Z)*-3-heptene-1-ol] was added. The reaction mixture was warmed to −30°C over 1 h, maintained at this temperature for 3 h, then quenched with 5 ml of a 25% ammonium acetate solution. The mixture was extracted with hexane (3×15 ml), and the combined extracts were washed with water (2×10 ml) and brine (10 ml), and then dried. All

Table 1 Chrysopids used for analyses of prothoracic gland secretions

Species[a]	Sex	No.	Date[b]	Location	Collection/Sampling Method
Chrysopa oculata	M	5	5 July	Beltsville, MD	Iridodial trap/Gland dissection
	M	4	27 Sept.	Beltsville, MD	Iridodial trap/Gland dissection
Chrysopa nigricornis	M	2	12 June	Spokane, WA	Iridodial lure/Gland dissection
	M	10	7 August	Spokane, WA	Fluorescent light[c]/Gland dissection
Chrysopa incompleta	M	8	24 Sept.	Byron, GA	Iridodial trap/Gland dissection
Chrysopa quadripunctata	M	2	31 May	Hyattsville, MD	Mercury vapor light/Thoracic extract
Chrysopa septempunctata	M	30	20 Aug.	Shenyang, China	Iridodial trap/ Thoracic extract
Ceraeochrysa cubana	M	5	19 April	Recife, Brazil	Laboratory colony/Thoracic extract
	F	5	19 April	Recife, Brazil	Laboratory colony/Thoracic extract
Chrysoperla rufilabris	M	5	8 Aug.	Hyattsville, MD	Mercury vapor light/Gland dissection
	F	4	8 Aug.	Hyattsville, MD	Mercury vapor light/Gland dissection
	M	3	22 Sept.	Byron, GA	Plant volatile trap/Gland dissection
***Chrysoperla* sp.**	M	5	17 April	Brasília, Brazil	Sweep netting/Thoracic extract
	F	5	17 April	Brasília, Brazil	Sweep netting/Thoracic extract
Plesiochrysa ramburi	F	1	8 Nov.	Brisbane, Australia	Sweep netting/Gland dissection
Mallada sp.	F	3	17 Nov.	Brisbane, Australia	Sweep netting/Gland dissection

[a] Determinations: *Co. oculata*, Dr. Oliver S. Flint, Jr., Section of Entomology, Smithsonian Institution, Washington D.C.; *Co. nigricornis*, Dr. N. D. Penny, Department of Entomology, California Academy of Sciences, San Francisco, USA; *Co. quadripunctata*, Dr. Catherine A. Tauber, Department of Entomology, Comstock Hall, Cornell University, Ithaca, NY, USA; *Co. septempunctata*, Dr. Baoyu Han, Tea Research Institute of Chinese Academy of Agricultural Sciences, Hangzhou, China; *Co. incompleta* and *Cl. rufilabris*, Dr. Ted E. Cottrell, USDA-ARS Southeastern Fruit & Nut Research Laboratory, Byron, GA; *Ceraeochrysa* and *Cl.* spp., Dr. Sérgio de Freitas, Universidade Estadual Paulista, Jaboticabal, São Paulo, Brazil; *Plesiochrysa* and *Mallada* spp., Dr. Shaun L. Winterton, Entomology Collection, Queensland Department of Primary Industries and Fisheries, Brisbane, Australia.

[b] All collected in 2007, except *Co. septempunctata* was collected in 2005.

[c] 13 watt Neolite® NL-13127 (Litetronics International, Inc., Alsip, IL, USA).

volatiles were removed *in vacuo*. Preparative thin layer chromatography (SiO_2, ethyl acetate/hexane, 1:7) gave *(Z,Z)*-4,7-tridecadiene (138 mg, 76%) and *(Z,Z)*-4,7-tridecadiene (6 mg, 3%). (*Z*)-4-Undecene, (*Z*)-4-tridecene, and (*Z*)-6-tridecene were synthesized by following the Wittig reaction procedure described above for *(Z,E)*-4,7-tridecadiene. The following reactions and products were obtained: the ylide of *n*-butyltriphenyl phosphonium bromide with heptanal produced 92% 4-undecenes (*Z*:*E,* 98:2); the ylide of *n*-butyltriphenyl phosphonium bromide with nonanal yielded 98% 4-tridecenes (*Z*:*E,* 93:7); and the ylide of *n*-heptyltriphenyl phosphonium bromide with hexanal gave 94% 6-tridecenes (*Z*:*E,* 95:5).

Chemical Analyses Lacewing extracts and chemical standards were analyzed in the splitless injection mode using an HP 6890 N gas chromatograph (GC) equipped with a DB-WaxETR column (0.25 μm film thickness, 30 m×0.25 mm ID; J & W Scientific, Folsom, CA, USA), with flame ionization detection. Hydrogen was used as the carrier gas, the injector and detector temperatures were 250°C and 300°C, respectively, and the column temperature was 50°C for 2 min, rising to 240°C at 10°C min^{-1}, and held for 10 min. GC-mass spectrometry (MS) was performed with an HP 6890 GC coupled to an HP 5973 mass selective detector using a DB-WaxETR column as above (except 60-m-long), programmed at 50°C for 2 min, rising to 230°C at 15°C min^{-1}, and held for 15 min.

Alkylthiolation for determining double bond position was accomplished using dimethyldisulfide (DMDS) derivatization as described by Zhu et al. (2000). For enhanced sensitivity in analyzing the reaction products, specific ion monitoring was used based on the *m/z* 61 ion $(CH_3SCH_2)^+$, which is present at 20–50% abundance in the spectra of all DMDS adducts (Leonhardt and DeVilbiss 1985).

Phylogenetic Analysis The Willi Hennig Society edition of "Tree analysis using New Technology" (TNT) software program was used to analyze the chemical data both qualitatively and quantitatively (Goloboff et al. 2008). Settings included a driven search for 500 replicates, set to find minimum length of one time per replicate. Search parameters included sectorial search, ratchet, drift, and tree fusing.

Results and Discussion

The results of our chemical analyses of lacewing PG secretions are summarized in Table 2. Including our analyses and those of others (Güsten and Dettner 1991; Zhu et al. 2000), the PG secretions of 23 species of green lacewings have now been analyzed, representing seven genera: those genera listed in Table 2, plus *Nineta* and *Chrysopidia* spp. (Güsten and Dettner 1991). Our results strongly support the conclusions of Güsten and Dettner (1991) that *(Z)*-4-tridecene is ubiquitous in green lacewing PG secretions, and other compounds are specific to one or only some genera, or some species within the *Chrysopa* (Table 2) as this genus now stands (Brooks and Barnard 1990; Brooks 1997).

We confirmed that the compound likely responsible for the fecal odor of *Chrysopa* spp. is skatole (3-methylindole) (Blum et al. 1973; Güsten and Dettner 1991), and extended the known distribution of this foul-smelling PG compound to two additional genera of green lacewings, *Plesiochrysa* and *Ceraeochrysa*. However, earlier reports of 1-tridecene in the prothoracic gland secretions of *Chrysopa oculata* (Blum et al. 1973; Zhang et al. 2004) and *Co. nigrispinus* (Zhang et al. 2006a) are here shown to be in error. Synthetic 1-tridecene did not coelute with the tridecene found in the PG secretions of these *Chrysopa* spp., whereas synthetic *(Z)*-4-tridecene did coelute with the natural products. The mass spectrum of 1-tridecene (not shown) exhibited a molecular ion (*m/z* 182) of about 15% the intensity of the base peak, whereas in the spectrum of *(Z)*-4-tridecene the molecular ion was nearly 60%. Furthermore, alkylthiolation of the prothoracic gland secretions of *Co. oculata* and *Co. nigrispinus* produced DMDS-derivatives showing the diagnostic mass spectral fragments for 4-tridecene (Zhu et al. 2000): m/z (%) 41(37), 55(47), 61(84), 69(86), 83(25), 103(65), 173(100), 229(4), and 276(M+, 21).

Beyond the universally present *(Z)*-4-tridecene, there are considerable differences in the distribution of other compounds among species, particularly for alkenes, alkadienes, and amides (Table 2). In three of the *Chrysopa* spp. we analyzed (*Co. nigrispinus*, *Co. incompleta*, and *Co. septempunctata*) PG secretions contained a 180 molecular weight

Table 2 Chrysopid prothoracic gland chemistry

Compound	Species[a] *P. r.*	*Co. n.*	*Co. i.*	*Co. s.*	*Co. o.*	*Co. q.*	*Ce. c.*	*Cl. r.*	*Cl.* sp.	*M.* sp.
(Z)-4-Undecene	40.17[b]	10.82	2.59					0.61		0.32
Undecadiene [c]	0.68									
(Z)-6-Tridecene		4.80	t [d]		t					
(Z)-4-Tridecene	1.35	26.33	40.39	13.98	62.10	53.63	19.90	89.49	100	90.39
(Z,Z)-4,7-Tridecadiene	26.42	27.99	22.65	20.86	3.50	t	1.49	9.49	t	5.59
Pentadecene [c]								0.19		0.35
Pentadecadiene [c]								0.22		t
Hexanoic acid							2.17			
Octanoic acid							1.04	t		
Nonanoic acid			1.48				0.66			
Skatole	16.92	23.17	24.87	32.28	22.29	40.37	49.78			
N-3-Methylbutylacetamide		6.44	6.05	32.86						
N-3-Methylbutylpropanamide	12.52	t	t							
3-hexanol							10.86			
2-hexanol							9.30			
Tridecanol	1.93									
Nonanal		t	3.52		7.00					
Decanal					5.10					
Benzaldehyde							4.82			

[a] *Plesiochrysa ramburi=P. r.*; *Chrysopa nigrispinus=Co. n.*; *Chrysopa incompleta=Co. i.*; *Chrysopa septempunctata=Co. s.*; *Chrysopa oculata= Co. o.*; *Chrysopa quadripunctata = Co. q.*; *Ceraeochrysa cubana = Ce. c.*; *Chrysoperla rufilabris=Cl. r.*; *Chrysoperla* sp.=*Cl.* sp.; *Mallada* sp.= *M.* sp.

[b] Percentages based on weighted mean gas chromatographic peak areas per compound per species.

[c] Standard not synthesized because insufficient natural product was available for further analysis; therefore, the identification is tentative based on mass spectral data; details in text.

[d] Trace≤0.1%.

compound, accounting for 20–30% of the excretion, which we thought to be a tridecadiene because the mass spectrum resembled that for *(Z)*-4-tridecene, but with the pattern of ions shifted two mass units lower. Indeed, the mass spectrum and retention time of the presumed lacewing tridecadiene matched the spectrum and retention time of synthetic *(Z,Z)*-4,7-tridecadiene, thus establishing the structure of the natural product. In *Plesiochrysa ramburi*, *(Z,Z)*-4,7-tridecadiene was much more abundant than *(Z)*-4-tridecene, and the eleven carbon alkene, *(Z)*-4-undecene [not 5-undecene as previously reported for *Co. nigrispinus* (Zhang et al. 2006a)], was identified as the major alkene constituent of the PG secretion based on mass spectral analysis of the DMDS derivative (Fig. 1A), and coinjection with the synthetic standard. The

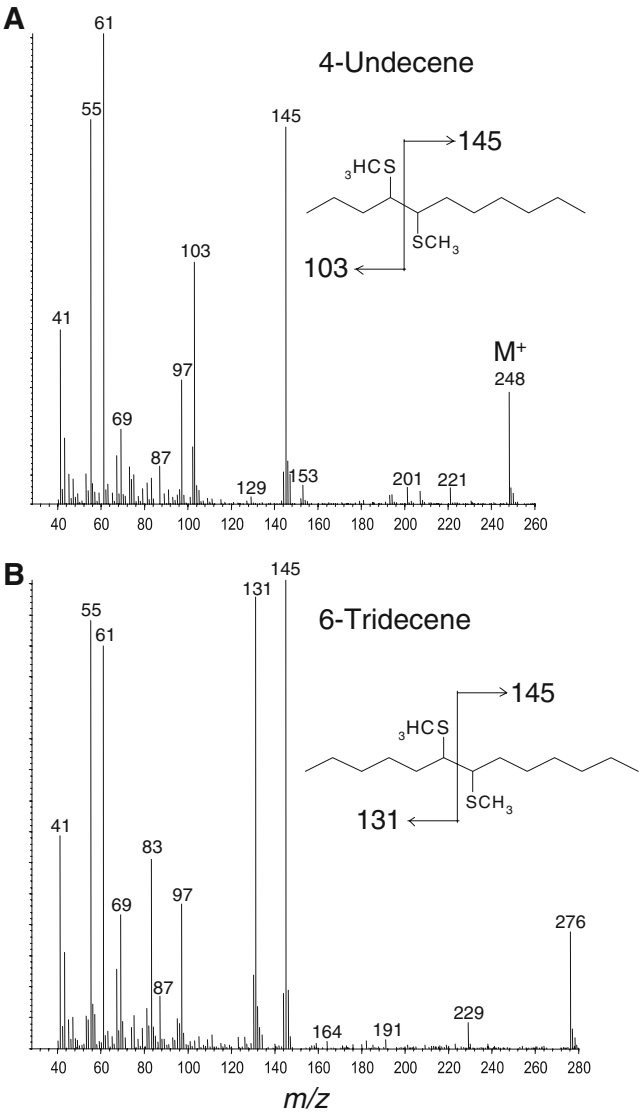

Fig. 1 Mass spectra of dimethyldisulfide derivatives of *(Z)*-4-undecene from the prothoracic gland (PG) secretion of *Plesiochrysa ramburi* (**A**), and *(Z)*-6-tridecene from the PG secretion of *Chrysopa nigricornis* (**B**)

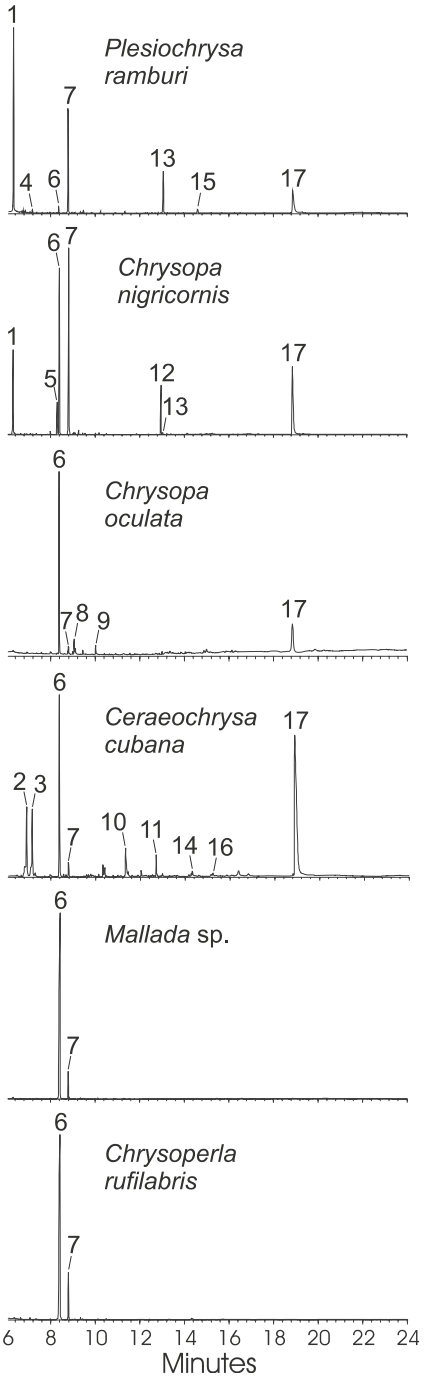

Fig. 2 Gas chromatograms of prothoracic gland extracts for representative species from the different genera and *Chrysopa* species groups analyzed [1=*(Z)*-4-undecene, 2=3-hexanol, 3=2-hexanol, 4=undecadiene, 5=*(Z)*-6-tridecene, 6=*(Z)*-4-tridecene, 7=*(Z,Z)*-4,7-tridecadiene, 8=nonanal, 9=decanal, 10=benzaldehyde, 11=hexanoic acid, 12=N-3-methybutylacetamide, 13=N-3-methylbutylpropanamide, 14=octanoic acid, 15=tridecanol, 16=nonanoic acid, and 17=skatole (3-methylindole)]

same group of *Chrysopa* spp. that express *(Z,Z)*-4,7-tridecadiene as a major PG secretion, also produced substantial quantities of N-3-methylbutylacetamide, a compound previously known as an allomone/pheromone in

cockroaches (Farine et al. 2002), yellowjacket wasps (Landolt and Heath 1987), and fruit flies (Bellas and Fletcher 1979). Curiously, the PG secretion of *P. ramburi* contained N-3-methylbutylpropanamide instead of the acetamide found in some of the *Chrysopa* species analyzed. Based on the mass spectrum of the DMDS derivative (Fig. 1B), as well as coinjection with the synthetic standard, *(Z)*-6-tridecene was also identified as a minor (*Co. nigricornis*) or trace (*Co. incompleta* and *Co. oculata*) PG component. Gas chromatograms of PG extracts for representative species from the different genera and *Chrysopa* species groups we examined are shown in Fig. 2.

Studies on pheromone biosynthesis in other insects suggest that *(Z)*-4-tridecene in lacewings may be biosynthesized from *(Z)*-9-octadecenoic (oleic) acid via successive cytosolic β-oxidation steps that remove four carbons, followed by decarboxylation (Jurenka 2004). *(Z)*-4-Undecene may be made from *(Z)*-9-hexadecenoic (palmitoleic) acid and tridecadiene most likely from *(Z,Z)*-9,12-octadecadienoic (linoleic) acid. Interestingly, *Chrysoperla carnea* is the only holometabolous insect known to synthesize linoleic acid (Cripps et al. 1986), and oleic and linoleic acids were the dominant fatty acids in abdominal extracts of *Co. oculata* in which fat body was present (JRA, unpublished data). Similarly, the biosynthesis of *(Z)*-6-tridecene may result from a single cycle of cytosolic β-oxidation of palmitoleic acid followed by decarboxylation.

The preliminary phylogenetic analysis of the semiochemical data (Table 2) resulted in an unresolved polytomy. Although the chemical signature of each of the species was distinct, the data set as a whole was more defined by autapomorphies than by synapomorphies. However, a grouping of *Chrysopa nigrispinus, Co. incompleta*, *and Co. septempunctata* was indicated based on the presence of N-3-methylbutylacetamide and the elevated expression of *(Z,Z)*-4,7-tridecadiene. Güsten and Dettner (1991) analyzed six *Chrysopa* species different from those we analyzed; all six of these *Chrysopa* were reportedly characterized by the presence of amides in their PG secretions (presumably the same acetamide we found). Thus, the *oculata-quadripunctata* grouping seems to represent a plesiomorphic clade distinct from those *Chrysopa* species that produce amides. Güsten and Dettner (1991) also noted that only *Chrysopa vididana* Schneider lacked skatole, which may be grounds to realign this species. Inclusion of other characters (e.g., morphological, molecular) in future analyses may provide further resolution.

Brooks and Barnard (1990) observed that "Chrysopidae is one of the largest and economically important families of the Neuroptera [~1200 species], [but] the classification of the family is confused." Much of the early chrysopid systematics was based on wing venation, which has been shown to be variable and unreliable (Brooks 1997). Today, quantitative computer analysis of DNA sequence data is the most powerful approach to deciphering phylogenetic relationships among insect taxa. Winterton and Freitas (2006) have applied this technique to the green lacewings, providing solid evidence that the Chrysopidae and Chrysopini are monophyletic, as well as clarifying the relationships of genera.

Apomorphic chrysopid PG semiochemicals are yet another set of quantifiable characters that may be incorporated with existing morphological, molecular, and courtship song data (Henry and Wells 2007) to further delineate relationships between green lacewings. Of course, many more species need to be analyzed chemically to lend strength to this approach. Preliminary efforts by one of us (JRA) have shown that it is feasible to obtain useful extracts of green lacewing PG secretions by briefly dipping live specimens in a minimal volume of solvent (unpublished data). At this point, the existing lacewing PG semiochemical data indicate that current *Chrysopa* spp. may be divisible based on the presence or absence of amides and skatole in their PG secretions. From a practical standpoint, the gas chromatographic "fingerprint" of chrysopid PG secretions (Fig. 2) appears to be a reliable means to help distinguish the common species of a region with the assistance of existing morphological keys (e.g., Penny et al. 2000). Certainly, one can quickly ascertain that a green lacewing that stinks is not a *Chrysoperla* species!

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In the Lepidoptera, the odorant-binding proteins were classified into pheromone-binding proteins (PBPs) and general odorant binding proteins (GOBPs) based on primary sequence homology (Vogt et al. 1991). Steinbrecht (1998) proposed that OBPs function to: (1) solubilize and carry olfactory signal molecules; (2) filter signals at the periphery; (3) stimulate the olfactory system; (4) clean the peri-receptor space; and (5) deactivate odorants, but the physiological function of OBPs is still under investigation. Specific binding to pheromones has been demonstrated for the PBPs in many species (Vogt et al. 1991; Du and Prestwich 1995; Feixas et al. 1995; Maida et al. 2000), and many arguments favor the involvement of PBPs in pheromone discrimination. In contrast, the role of GOBPs is still unclear. To date, several GOBP genes have been cloned (Maíbèche-Coisné et al. 1998; Ishida et al. 2002b; Ban et al. 2003; Calvello et al. 2005). Surprisingly, binding properties of GOBP2 have been demonstrated for the major pheromonal compound in several insect species (Vogt and Riddiford 1981; Feng and Prestwich 1997).

The diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), is a significant pest of vegetables. Integrated pest management (IPM) strategies are being developed against this species, including olfactory-mediated behavioral manipulation. In order to exploit the molecular mechanism of the perception of volatile cues by *P. xylostella*, we investigated its repertoire of OBPs by using polymerase chain reaction (PCR) strategies and compared the GOBPs with previously reported sequences from other members of the Lepidoptera.

Methods and Materials

Insect Rearing and Collection of Tissues *Plutella xylostella* larvae were cultured indoors under a 16:8 h (L:D) light cycle at 25±1°C, 60–70% RH, and fed with Chinese cabbage, *Brassica oleracea var. capitata* L. (Brassicaceae) until pupation. Sexed pupae were kept inside glass tubes in an environmental chamber at a 16:8 h (L:D) light cycle at 25±1°C, 60–80% RH until the moths emerged. To obtain mated females, newly emerged male and female moths were paired individually in glass tubes and allowed to mate.

Antennae were collected by excising the tissue at the base from 2-d-old male and female moths and immediately transferring them to Eppendorf tubes immersed in liquid nitrogen. The same procedure was used for collecting the head (without antennae), wings, abdomen, and legs. All tissues were stored at −75°C until used experimentally.

RNA Extraction and cDNA Synthesis Total RNA was extracted from 200 antennae from sexually mature *P. xylostella* males and females by using TRIpure LS Reagent (BioTeke Corporation, Beijing, China). Single-stranded cDNA was synthesized from 1 μg of RNA with the MBI RecertAid First Strand cDNA Synthesis Kit (MBI Fermentas, Glen Burnie, MD, USA) according to the manufacturer's instructions, and single-stranded 3′RACE-ready-cDNA and 5′RACE-ready-cDNA were synthesized from 1 μg of RNA with the TaKaRa RACE cDNA Amplification Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions.

Degenerate PCR for Isolation of PxylGOBP1 and PxylGOBP2 Gene Fragments The cDNAs that encode pheromone binding proteins of *P. xylostella* were amplified from antennal cDNA of males. Degenerate primers corresponding to amino acid consensus regions of several GOBP1s and GOBP2s from different species of Lepidoptera were used for PCR amplification of *P. xylostella* cDNA templates: GOBP1-forward (5′-ATGTCACGCTAGGCT TCGG-3′) and GOBP1-reverse (5′-AGGATACGCCAG CAGTGGT-3′); GOBP2-forward (5′-GAGTTCMARCACT TCTGGMG-3′) and GOBP2-reverse (5′-AAGCAV GCWGCNACYTTNAC-3′). PCR was carried out with 2 μl cDNA, 15 pmol of each primer, 10 nmol of each deoxynucleoside triphosphate (dNTP), and one unit of Taq DNA polymerase (MBI Fermentas) in the supplied buffer, giving a final concentration of 2.0 mM $MgCl_2$ in 25 μl. Cycle conditions were as follows: initial denaturation at 95°C for 3 min; 35 cycles of 94°C for 1 min, 54°C for 40 sec, and 72°C for 1 min; final extension at 72°C for 10 min. All amplification products were purified from 1.5% agarose gels using a gel extraction kit (Sunny Biotechnologies, Shanghai, China). The purified fragment was linked with pMD-18T vector (TaKaRa) and sequenced.

3′RACE-PCR 3′RACE (rapid-amplification of cDNA ends) amplifications were conducted on 2 μl of *P. xylostella* 3′ RACE-ready cDNA, with sense gene-specific primers deduced from the sequences obtained following 3′RACE outer and inner PCR amplifications of GOBP1 (GOBP1 3′ RACE outer primer: 5′-GAACATTGTCGGGAGCAGTC-3′ and GOBP1 3′RACE inner primer: 5′-GACAACACG CACAAGTTCATAC-3′); GOBP2 (GOBP2 3′RACE outer primer: 5′-CCACTGCATGAGCCGCTACTTC-3′ and GOBP2 3′RACE inner primer: 5′-AGGCTGATCCACAA CTGTGAG-3′) and with an antisense 3′RACE outer and inner primers (TaKaRa). The 50-μl amplification mix was prepared according to the TaKaRa cDNA protocol with Taq polymerase mix (MBI Fermentas). 3′RACE outer PCR was performed under the following conditions: 3 min at 95°C, followed by 5 cycles of 30 sec at 94°C and 1 min at 72°C; 5 cycles of 30 sec at 94°C, 30 sec at 70°C, and 1 min at 72°C; 30 cycles of 30 sec at 94°C, 30 sec at 68°C, and 1 min at 72°C, and 3′RACE inner PCR were performed in the following conditions: 3 min at 95°C, followed by 30

cycles of 30 sec at 94°C and 1 min at 72°C; 5 cycles of 30 sec at 94°C, 30 sec at 70°C, and 1 min at 72°C; 30 cycles of 30 sec at 94°C, 30 sec at 68°C, and 1 min at 72°C.

5′RACE-PCR 5′RACE amplifications were conducted on 2 μl of *P. xylostella* 5′RACE-ready cDNA, with sense gene-specific primers deduced from the sequences obtained following 3′RACE outer and inner PCR amplifications of GOBP1 (GOBP1 5′RACE outer primer: 5′-GCCCAGCAG GTTGAAGTAGC-3′ and GOBP1 5′RACE inner primer: 5′-GCTGCGACTGCTCCCGACAA-3′); GOBP2 (GOBP2 5′RACE outer primer: 5′-GATGTCGTCGTACTT CTTCTCA-3′ and GOBP2 5′RACE inner primer: 5′-AAG CTCTTGATGTAGTCGTGCA-3′) and with antisense 5′ RACE outer and inner primers (TaKaRa). The 50-μl amplification mix was prepared according to the TaKaRa cDNA protocol with Taq polymerase mix (MBI Fermentas). PCRs were performed as described for the 3′RACE-PCR.

Cloning and Sequencing After gel extraction (Sunny Biotechnologies), amplified cDNAs were cloned into a pMD-18T vector (TaKaRa). Recombinant plasmids were isolated by using the Plasmid Mini kit (Sunny Biotechnologies), and custom sequenced. Database searches were performed with the BLAST program (NCBI), protein analyses with MWCALC (Infobiogen) and SignalP (Nielsen et al. 1997), and sequence alignment with CLUSTALW (NPS@IBCP).

Phylogenetic Analysis with Other Lepidoptera GOBPs Several Lepidoptera GOBP sequences were retrieved from GenBank for phylogenetic analysis. Amino acid sequences of GOBPs without the signal peptide sequence were aligned by using the Multalin program (Corpet 1988). Maximum parsimony was used to build a strict consensus tree with MEGA4.0 software. Branch support was assessed by bootstrap analysis based on 1,000 replications.

Tissue Specificity of PxylGOBPs Tissue specificity was assessed by reverse transcription (RT)-PCR. RT-PCR templates were the cDNA from antennae and other tissues of male and female moths. Specific primer pairs (PxylGOBP1-forward: 5′-CACCATGACAACACGCACAAG-3′, PxylGOBP1–reverse: 5′-ACTCTGCCATCAGCATC TCCA-3′) (PxylGOBP2-forward: 5′-GAAGAATCCGGCC TATCC-3′, PxylGOBP2–reverse: 5′-CAGCCTCACCA TCGTCTC-3′) were derived from the cDNA sequences. For testing the integrity of the cDNA templates, a control primer pair (PxylActin-forward: 5′-GCGACTTGACCGA CTACC-3′, PxylActin–reverse: 5′-GGAATGAGGGCTGG AACA-3′) from the coding region of the *P. xylostella* actin gene (GenBank AB282645) was used. PCR began at 95°C for 3 min, then 35 cycles at 94°C for 30 sec, 54°C for 60 sec, and 72°C for 1 min, with a final 10 min elongation step at 72°C.

Expression Profiling of PxylGOBP1 and PxylGOBP2 Based on Real-Time PCR For the experiment examining *PxylGOBP1* and *PxylGOBP2* expression through a larval molt cycle, we used 200 eggs; 100 first instar larvae; 50 s and third instar larvae; 100 heads of male and female fourth instar larvae; and 20 male and female pupae. For expression profiling across ages of adult moths, the antennae of 0, 4, 8, and 16 h-old adult virgin and mated moths were used. To obtain mated moths, newly emerged male and females were paired individually in glass tubes and allowed to mate and were collected after 16 h. Total RNA from all of the above material from 100 moths was extracted, and cDNA was synthesized according the methods described above. Specific primer pairs for cloning *PxylGOBP1*, *PxylGOBP2*, and *Pxylactin* were the same as the primer pairs for RT-PCR, and were expected to amplify 209 bp, 219 bp, and 252 bp fragments, respectively. Real-time PCR was performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) by using SYBR green dye bound to double strand DNA at the end of each elongation cycle (QuantiTect SBGR Green PCR kit; Qiagenn, Beijing, China).

The thermal cycling conditions for real-time PCR were 15 min at 95°C, followed by 40 cycles of 15 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C. PCR reactions were performed in triplicate, and data were processed by using the relative quantification method. The cDNA sample from male *P. xylostella* antenna was diluted to C0 (1, 4, 16, 64, and 256) times, and the CT values of *PxylGOBP*s and *PxylActin* were measured by real-time PCR. The standard curve was performed by CT and logC0 (the slope K and intercept B), and the relative values were measured as $10^{\Delta CT}$ (where ΔCT = CTPxylGOBPs[-(CT-B)/K] – CTPxylActin[-(CT-B)/K)]. Background data for this quantification have been provided (Rn vs Cycle of Real-time PCR and standard curve in Supplemental Data Figs. 1, 2, 3, 4, 5, 6).

Results

Molecular Cloning and cDNA Sequencing of P. xylostella GOBP1 and GOBP2 The RT-PCR approach performed with two pairs of degenerate primers identified two cDNA fragments 317 bp (*GOBP1*) and 260 bp (*GOBP2*), respectively. By RACE-PCR strategies, two full-length cDNAs, encoding potential GOBPs, were cloned from *P. xylostella*. They both contained an open-reading frame encoding two putative proteins of 168 and 163 amino acids, which we have named PxylGOBP1 and PxylGOBP2, respectively. The names were assigned according to the high identity of these proteins with proteins from these

classes in BLAST searches. The sequences for PxylGOBP1 and PxylGOBP2 have been deposited in GenBank under the accession numbers ABY71034, and ABY71035, respectively. The deduced amino acid sequences for both proteins possess a putative signal sequence of 24 (PxylGOBP1) and 22 (PxylGOBP2) amino acids (Fig. 1). Mature predicted PxylGOBP1 protein consisted of 144 amino acids with a molecular mass of 16,922 Da and a pI of 4.92, whereas PxylGOBP2 consisted of 141 amino acids with a molecular mass of 16,356 Da and a pI of 5.03. Thus, the two predicted mature proteins are small, likely soluble, likely secreted, and both have an acidic isoelectric point. Moreover, both contained six cysteines in conserved positions when aligned with other known GOBPs: positions C^{19}, C^{50}, C^{54}, C^{97}, C^{108}, and C^{117} (Fig. 2).

We aligned the mature amino acid sequences of PxylGOBPs with GOBPs from *Bombyx mori* (Lepidoptera: Bombycidae) and 14 other species of Lepidoptera (Figs. 2 and 3). PxylGOBPs have conserved motifs characteristic of Lepidoptera GOBPs, including the six cysteine residues that form three disulfide bridges, and the hydrophobic domains. The two PxylGOBPs can be classified into different GOBP groups (group1 and group 2) by phylogenetic analysis, and PxylGOBP1 was closest phlylogenetically to the corresponding GOBP from *B. mori* (Fig. 4). Similarity analysis on the amino acid sequences of GOBPs further confirmed the phylogenetic result. The two PxylGOBPs share only 50% identity, but are more identical to GOBPs of the same group from other moth species. PxylGOBP1 displays high identities with BmorGOBP1 (61.18%), HassGOBP1 (59.76%), AsegGOBP1 (59.76%), and SlitGOBP1 (63.31%), whereas PxylGOBP2 is more identical with BmorGOBP2 (65.06%), HassGOBP2 (72.29%), AsegGOBP2 (65.66%), and SlitGOBP2 (70.48%).

Tissue Specificity of PxylGOBPs RT-PCR experiments were performed by using specific primers to determine the tissue distribution of *PxylGOBP1* and *PxylGOBP2* (Fig. 4). RT-PCR products of the predicted size were observed exclusively in reactions with antennal cDNA of both sexes. No specific product was observed with head (without antennae), leg, abdomen, or wing. The integrity of the cDNA templates prepared from different tissues was verified by primers specific for the actin gene. In all cDNA preparations, an Actin amplification product of the correct size was amplified. Therefore, *PxylGOBPs* are only expressed in antennae.

GOBPs Expression Patterns in P. xylostella Real-time PCR was performed to compare the transcript levels of *PxylGOBPs* from antennae of male to female moths and through a larval molt cycle. For each sex and each time, cDNA was synthesized from mRNA isolated from pools of 200 antennae.

PxylGOBPs were expressed throughout the life cycle of *P. xylostella* (including egg, larva, pupa, and adult) (Figs. 5 and 6, CT data shown Supplemental Data Tables 1 and 2). The transcription levels of both *PxylGOBP1* and *PxylGOBP2* were very low in egg, larval, and pupa stages compared to adult moth, just about 0.1‰ (Figs. 5 and 6). The transcription levels of both *PxylGOBP1* and *PxylGOBP2* were lowest in eggs of whole period; which in first

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1    ATGGAGCGCAGGTGGTGTCTGCTGGTGCTGGCGGCGGCGGCGGCGGCGGGGCTGCCCGGG
1     M  E  R  R  W  C  L  L  V  L  A  A  A  A  A  A  G  L  P  G

61   GTGGTGCGGGGGACGGTCGAGGTCATGAAGGACGTCACGCTCGGGTTCGGGGAGGCGCTT
21    V  V  R  G  T  V  E  V  M  K  D  V  T  L  G  F  G  E  A  L

121  GAGCATTGTCGGGAGCAGTCGCAGCTAACAGAAGAGATGATGGAAGAGTTCTACCACTTC
41    E  H  C  R  E  Q  S  Q  L  T  E  E  M  M  E  E  F  Y  H  F

181  TGGCGGGAAGACTTCAAGTTCGAGGCTCGCGCCGTGGGCTGCGCCATCCACTGCATGAGC
61    W  R  E  D  F  K  F  E  A  R  A  V  G  C  A  I  H  C  M  S

241  CGCTACTTCAACCTGCTGGGCGAGCAGCAGCGCATGCACCATGACAACACGCACAAGTTC
81    R  Y  F  N  L  L  G  E  Q  Q  R  M  H  H  D  N  T  H  K  F

301  ATACAGAGCTTCCCTAATGGCGAAGTCCTCTCGCACCAGATGGTGGGCATCATCCACACG
101   I  Q  S  F  P  N  G  E  V  L  S  H  Q  M  V  G  I  I  H  T

361  TGCGAGCAGCAGCACGACGCGGAGACGGACGACTGCTGGCGGATCCTGCGAGTGGCGGAG
121   C  E  Q  Q  H  D  A  E  T  D  D  C  W  R  I  L  R  V  A  E

421  TGCTTCAAGCGGGAGAGCCAGGCGCAGGGGCTGGCCCCGAGCATGGAGATGCTGATGGCA
141   C  F  K  R  E  S  Q  A  Q  G  L  A  P  S  M  E  M  L  M  A

481  GAGTTTATTATGGAGGCCGATGTGTGA
161   E  F  I  M  E  A  D  V  *
```

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1    ATGGCGTCAGTGTGGAGTCTAGTTGTCTGTGGTCTGATGATGGCGGCGCTTCCGGCCGCG
1     M  A  S  V  W  S  L  V  V  C  G  L  M  M  A  A  L  P  A  A

61   AGGGGCACTGCAGAGGTCATGTCGCATGTCACTGCGCACTTTGGAAAGACCCTGGAAGAA
21    R  G  T  A  E  V  M  S  H  V  T  A  H  F  G  K  T  L  E  E

121  TGCAGGGAAGAATCCGGCCTATCCGGCGAAATAATGGAAGAGTTCCACCACTTCTGGCGC
41    C  R  E  E  S  G  L  S  G  E  I  M  E  E  F  H  H  F  W  R

181  GAGGACTTCGAGGTGGTGCACCGCGAGCTGGGCTGCGCCATCATCTGCATGAGCAACAAG
61    E  D  F  E  V  V  H  R  E  L  G  C  A  I  I  C  M  S  N  K

241  TTCCAGCTGATGCAGGACGACGCGCGCATGCACCACGAGAACATGCACGACTACATCAAG
81    F  Q  L  M  Q  D  D  A  R  M  H  H  E  N  M  H  D  Y  I  K

301  AGCTTCCCCAAGGGTGACCTCCTCTCAGAGACGATGGTGAGGCTGATCCACAACTGTGAG
101   S  F  P  K  G  D  L  L  S  E  T  M  V  R  L  I  H  N  C  E

361  AAGAAGTACGACGACATCGACGACGAGTGCAGCCGCGTGGTGAAGACTGCCGCCTGCTTC
121   K  K  Y  D  D  I  D  D  E  C  S  R  V  V  K  T  A  A  C  F

421  AAGAAGGATGCTCAGGCCGAGGGCATCGCCCCCGAGCTCACCATGATCGAGGCCGTGCTG
141   K  K  D  A  Q  A  E  G  I  A  P  E  L  T  M  I  E  A  V  L

481  GAGAAGTACTGA
161   E  K  Y  *
```

Fig. 1 Nucleotide and deduced amino acid sequences of pxylGOBP1 (*left*) and pxylGOBP2 (*right*) cDNAs from antennae of the diamondback moth, *Plutella xylostella* (GenBank: ABY71034 and ABY71035). The stop codons are indicated by an *asterisk*. Putative signal peptides at the N-termini are *underlined*

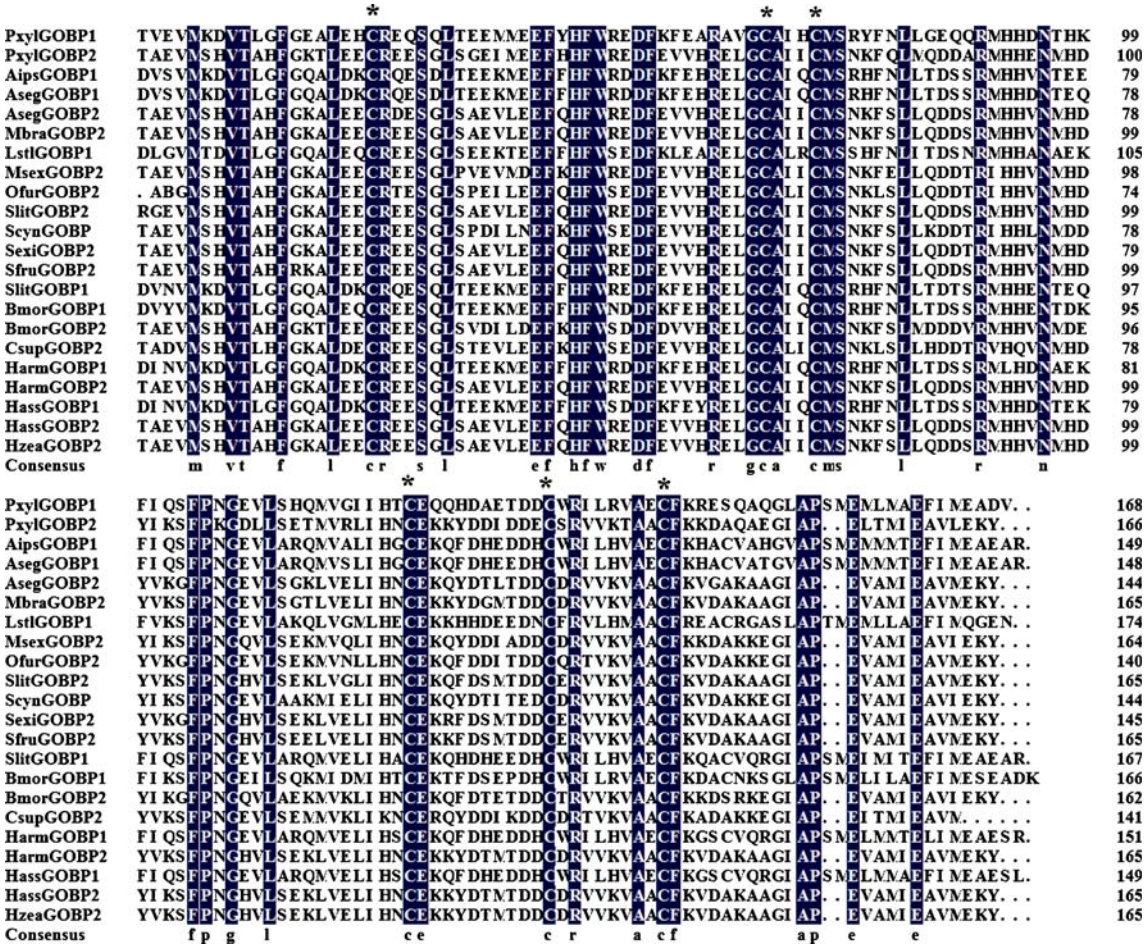

Fig. 2 Comparison of the predicted amino acid sequences of PxylGOBP1 and PxylGOBP2 from antennae of male *Plutella xylostella* with GOBPs from other Lepidoptera. The six conserved cysteines are marked by an asterisk. GenBank accession numbers—PxylGOBP1: ABY71034; PxylGOBP2: ABY71035; AipsGOBP1: ABI24160; AsegGOBP1: ABI24159; AsegGOBP2: ABI24161; BmorGOBP1: NP_001037496; BmorGOBP2: NP_001037498; CsupGOBP2: ABD98823; HarmGOBP1: AAL09821; HarmGOBP2: CAC08211; HassGOBP1: AAW65076; HassGOBP2: AAQ54909; HzeaGOBP2: AAG54078; LstiGOBP1: ACB47481; MbraGOBP2: AAC05703; MsexGOBP2: AAG50015; OfurGOBP2: ABG66419; ScynGOBP: BAF91328; SexiGOBP2: CAC12831; SfruGOBP2: AAT74555; SlitGOBP1: ABM54823; and SlitGOBP2: ABV32167

instar were highest of whole larval period, and the transcription level of *PxylGOBP1* was about double that of other larval periods; the transcription levels of *PxylGOBP1* in male pupa were higher than in female pupa; the transcription levels of *PxylGOBP2* decreased after pupation. The transcription levels of *PxylGOBPs* increased sharply after eclosion. The transcription levels of *PxylGOBPs* in male antenna were higher than those in female antenna. Mated individuals showed much higher levels of *PxylGOBPs* expression at 16 h post emergence than their unmated counterparts. The transcription levels of *PxylGOBPs* in 4 h-old moth antenna were the lowest in males, whereas they were highest in females. The transcription levels of *PxylGOBP1* from antennae were lower than *PxylGOBP2* in both male and female moths, and the amounts of *PxylGOBP1* mRNA were about 20% of *PxylGOBP2* mRNA (Fig. 6).

Discussion

In *P. xylostella*, no GOBPs had been described prior to our study. Deciphering the GOBP repertoire in this species provides the opportunity to relate GOBP expression with the functional types of sensilla. Here, we have cloned two cDNAs that encode proteins that can be classified in the lepidopteran GOBP family. Indeed, the deduced amino acid sequences present all of the typical features of OBPs: (1) they are small hydrophilic proteins with acidic isoelectric points; (2) they are destined for secretion, as demonstrated by the occurrence of a signal peptide at the N-termini; (3) they possess six cysteines in positions conserved across OBPs (Pelosi et al. 2006). The deduced proteins were grouped in the GOBP families, according to sequence homologies with lepidopteran GOBPs and phylogenetic

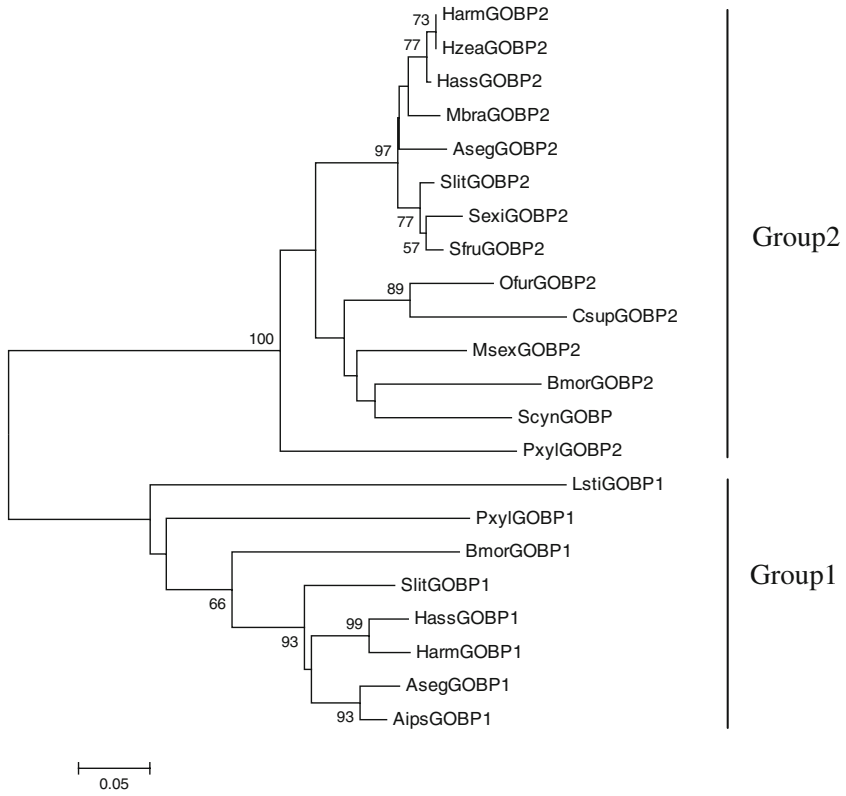

Fig. 3 Phylogenetic analysis of PxylGOBPs amino acid sequences with various lepidopteran GOBPs. GenBank accession numbers are listed in Fig. 2. Bootstrap support values (in percent) based on 1,000 replicates are indicated. Analysis indicates two groups (Grp1 and Grp2), as defined by Picimbon and Gadenne (2002)

analyses, and thus have been named PxylGOBP1 and PxylGOBP2.

Phylogenetic analysis showed that Lepidoptera GOBPs are classified into two distinct groups (Fig. 3): PxylGOBP1 belongs to group1 and PxylGOBP2 belongs to group 2. Group 1 contains PxylGOBP1, bmorGOBP1, HassGOBP1, slitGOBP1 etc.; group 2 includes PxylGOBP2, bmorGOBP2, HassGOBP2, slitGOBP2, etc. A more meaningful phylogenetic tree of insect GOBPs can be constructed when more GOBPs become available from a larger number of species. Insect OBPs have also been classified into long chain (~160 aa), medium chain (~120 aa), and short chain (~110 aa) classes, which relates to their potential structure and function (Pesenti et al. 2008). According to this hypothesis, PxylGOBP1 (168 aa) and PxylGOBP2 (163 aa) belong to the long chain class, and this is useful to know for future functional studies of these proteins.

OBPs are presumed to mediate the delivery of odorants to specific receptor proteins embedded in the dendritic membrane (Vogt et al. 1999). Steinbrecht (1998) proposed that OBPs function to solubilize and carry olfactory signal molecules, but the physiological function of OBPs is still

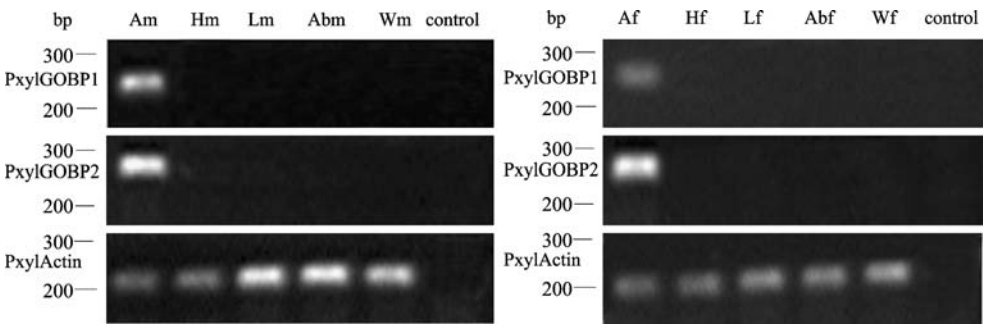

Fig. 4 Tissue-specific expression of *PxylGOBPs*. RT-PCRs were performed by using RNAs isolated from the different tissues. Amplification products were analyzed on agarose gels and visualized by UV illumination after staining with ethidium bromide. Based on the primer design, the sizes of the expected PCR-products were 209 bp for *PxylGOBP1*, 219 bp for *PxylGOBP2*, and 252 bp for *Actin* (control). *Am*: Male antenna, *Af*: female antenna, *Hm*: male head (without antenna), *Hf*: female head (without antenna), *Abm*: male abdomen, *Abf*: female abdomen, *Lm*: male leg, *Lf*: female leg, *Wm*: male wings, *Wf*: female wings, control: no-template (negative control) ensures the specificity of the amplifications. The position of molecular weight markers (*bp*) is indicated on the *left* side

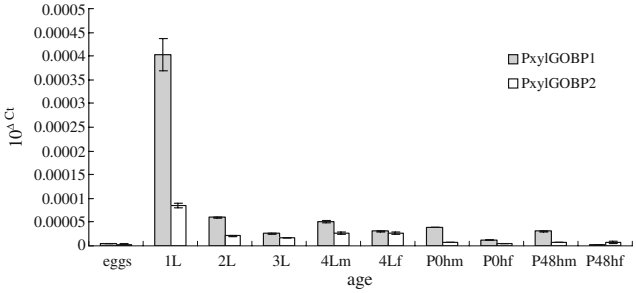

Fig. 5 Relative quantification of expression of *PxylGOBP1* and *PxylGOBP2* amplified through a larval molt cycle by real-time PCR. 1 L: first larval instar; 2 L: second larval instar; 3 L: third larval instar; 4 L: fourth larval instar; P0hm: 0 hr male pupa; P0hf: 0 hr female pupa; P48hm: 48 hr male pupa; and P48hf: 48 hr female pupa

under investigation. There are several studies that support the role of PBP's in pheromone detection (Ljungberg et al. 1993; Schneider et al. 1998; Fan et al. 2003; Xu et al. 2005), but the functional role of GOBPs is still unclear. GOBPs were first presumed to mediate the delivery of plant volatiles, since they are localized in *B. mori* and *A. polyphemus* in sensilla basiconica (Laue et al. 1994; Heinbockel and Kaissling 1996), which respond to food or host-plant odors (Priesner 1979). Surprisingly, binding properties of GOBP2 were only shown for the major pheromonal compound in *A. polyphemus* (Vogt and Riddiford 1981). Recombinant GOBP2 from *Manduca sexta* also showed strong affinity for the major pheromonal compound of this species, *trans*-6, *cis*-11-hexadecenyl acetate [(*E*)-6-(*Z*)-11-16:Ac], binding of which is only displaced by very high concentrations of plant odors (Feng and Prestwich 1997). In *Mamestra brassicae*, a recombinant GOBP2 was expressed in *Escherichia coli* and gave positive binding with three tritiated pheromone analogs (Maíbèche-Coisné et al. 1998). However, the native form of this protein (purified from male antennae) did not show affinity for the pheromone components, but displayed high specific affinity for *cis*-11-hexadecenol, an antagonist to pheromone-mediated male attraction (Jacquin-Joly et al. 2000).

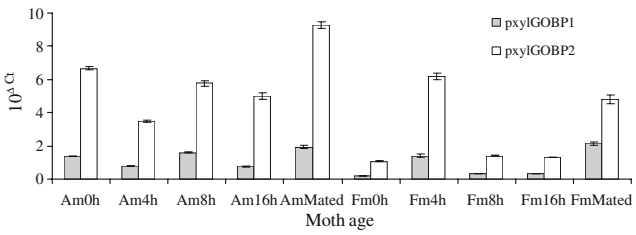

Fig. 6 Relative quantification of expression of *PxylGOBP1* and *PxylGOBP2* amplified at separate times by real-time PCR. *Am*: Male antenna; *Af*: female antenna; 0 hr, 4 hr, 8 hr, and 16 hr and mated refers to the adult at 0 hr, 4 hr, 8 hr, and 16 hr after eclosion and mating

The transcription levels of *P. xylostella* GOBP genes differed among mated and unmated male and female moths, and also correlated by age. This result agrees with the report that mated females were more responsive to three green leaf volatiles (GLVs) viz., (*Z*)-3-hexenyl acetate, (*E*)-2-hexenal, and (*Z*)-3-hexene-1-ol than males or virgin females (Reddy and Guerrero 2000). In fact, many moths have showed a plasticity of olfactory-guided behavior not only dependent on the nature of the chemical, but that might change with the physiological status (e.g., age/hormone or mating status) of the individual (Anton et al. 2007). For example, mated females, but not virgin females, of *Amyelois transitella* (Walker) flew upwind in an odor plume containing almond volatiles (Phelan and Baker 1987). In female *Lobesia botrana*, wind tunnel experiments with various grapevine plant parts as odor sources, demonstrated that only mated females were attracted. Virgin females never showed any upwind flight behavior in response to any of the plant parts used (Masante-Roca et al. 2007). Similar phenomena were found in adult female *Manduca sexta* (Mechaber et al. 2002). Gypsy moth, Lymantria dispar, PBPs discriminate among structurally related odorants (Plettner et al. 2000; Kowcun et al. 2001). Laughlin et al. (2008) also found that OBP-ligand complexes of a very specific conformation activate the receptor and that "locking" the OBP (through a mutation) into the conformation can cause the receptor and sensillum activation without the presence of a ligand. However the electrophysiological responses to a specific ligand do not correlate with the binding affinity of the gypsy moth PBPs for that ligand (Honson et al. 2003). In the future, we plan to investigate the relationship between the transcription of GOBP genes and behavioral plasticity in *P. xylostella*.

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pest control and for basic research of species-specific pheromonal communication. We report here the identification of compounds in pheromone gland extracts of female *S. haitangvora*, and the male responses to blends of different components and ratios in the field.

Methods and Materials

Insects Late stage larvae of *S. haitangvora* were collected by excavating them from infested trunks of apple trees during May and June of 2008 at Suwon (37.2°N,127.1°E), Korea. They were individually reared in capped plastic cups (30 ml) on artificial diet (Bio-serv, Frenchtown, NJ, USA) and maintained at 23°C under a 16L:8D photoperiod. After eclosion, the sexes were separated based on distinctive hair tufts (Arita et al. 2004). Moths were provided with a cotton pad soaked with a 5% (*w*/*v*) sucrose solution as food.

Chemicals Synthetic pheromone standards used were purchased from Pherobank (Wageningen, The Netherlands). Isomeric purity of these compounds exceeded 99%. C23–C28 straight-chain hydrocarbons were obtained from Sigma-Aldrich (Missouri, USA).

Pheromone Extraction As female *S. haitangvora* exhibited calling behavior (extending their ovipositors) during photophase, pheromone gland extracts were taken from 1- to 2-d-old females at 4 h into the photophase. The pheromone gland of each female was extruded by applying gentle pressure to the abdomen, excised with fine forceps, and extracted in 10 μl hexane containing 10 ng of tridecyl acetate (13:OAc) as an internal standard into a 0.3-ml conical vial (Wheaton, Millville, NJ, USA) for 10 min at room temperature. The extract was transferred to a new vial and stored at −80°C until analysis.

Chemical Analysis Pheromone gland extracts were analyzed on an Agilent 6890N GC interfaced to an Agilent 5973 mass-selective detector. Samples were run on DB-23 and DB-Wax columns (30 m×0.25 mm ID, 0.25 μm film thickness, J&W Scientific, Folsom, CA, USA) in the splitless mode. Injector and detector temperature were 250°C. Helium was used as carrier gas (1 ml/min). The GC oven temperature was programmed from 80°C (1 min hold) to 220°C at 10°C/min and held for 10 min. The ionization voltage was 70 eV. The ion source temperature was 230°C. Components in gland extracts were identified tentatively by comparison of their mass spectra with the mass spectra library (Wiley-NIST, Hoboken, NJ, USA); and identifications were confirmed by comparison of retention indices (RI; relative to alkane standards, Van den Dool and Kratz 1963), and mass spectra with those of authentic standards on two different columns. The quantity of each component was estimated by comparing its GC peak area with that of the internal standard (13:OAc).

Field Experiments Field experiments were conducted during August–September of 2008 in apple orchards at Suwon, Korea. Sticky delta traps (Green Agro Tech, Korea) baited with rubber septa (Aldrich Chemical Co., Milwaukee, WI, USA) impregnated with test chemicals in hexane were hung on branches 1–1.5 m above ground level. All field tests employed a complete randomized block design with four replicate blocks. The distance between traps within a block was at least 10 m. Trapped moths were counted and removed daily.

Experiment 1 investigated the attraction of *S. haitangvora* males to the two major components *Z*3,*Z*13-18:OAc and *E*2,*Z*13-18:OAc singly and in binary blends. Experiment 2 was conducted to test the individual effects of seven minor components identified in female gland extracts; *Z*9-16:OAc, *Z*11-16:OAc, *Z*9-18:OAc, *Z*13-18:OAc, *E*3,*Z*13-18:OAc, *Z*3,*Z*13-18:OH, and *E*2,*Z*13-18:OH as possible synergists, by using standard baits of the two major components. Finally, experiment 3 compared the relative attractiveness of different doses of a 1:1 blend of *Z*3,*Z*13-18:OAc and *E*2,*Z*13-18:OAc. Trap catch data (x) were transformed to log (x+1) and submitted to one-way analysis of variance. Means were compared by Tukey's test at α=0.05 (SAS Institute Inc. 2004).

Results

Chemical Analysis GC-MS analysis of pheromone gland extracts of female *S. haitangvora* revealed the two major and seven minor components, as well as henicosane, tricosane, pentacosane, and heptacosane (Fig. 1). The mass spectrum of peaks 1 and 2 had diagnostic fragment ions *m/z* 282 (M^+), 222 (M^+-CH_3COOH), and 61 ($CH_3COOH_2^+$), suggesting hexadecenyl acetate. Peaks 3 and 4 showed diagnostic ions *m/z* 310 (M^+), 250 (M^+-CH_3COOH), and 61 suggestive of octadecenyl acetate. For identification of the monoene acetates, the retention indices (RIs) of the *E* and *Z* isomers of hexadecenyl and octadecenyl acetates in which the double bond position ranged from carbon 7 to 13 were calculated on the two GC columns. The RIs of peak 1 and the *Z*9-16:OAc standard were the same on the DB-Wax and DB-23 columns (RIs of 2,342 and 2,386, respectively), and peak 2 coincided with *Z*11-16:OAc in GC retention times on both columns (RIs of 2,354 and 2,400, respectively). The calculated RIs of peak 3 and the *Z*9-18:OAc standard were the same on the DB-Wax and DB-23 columns (RIs of 2,529 and 2,594, respectively), and peak 4 had RIs of 2,555 and 2,620, respectively, the same as those of *Z*13-18:OAc standard.

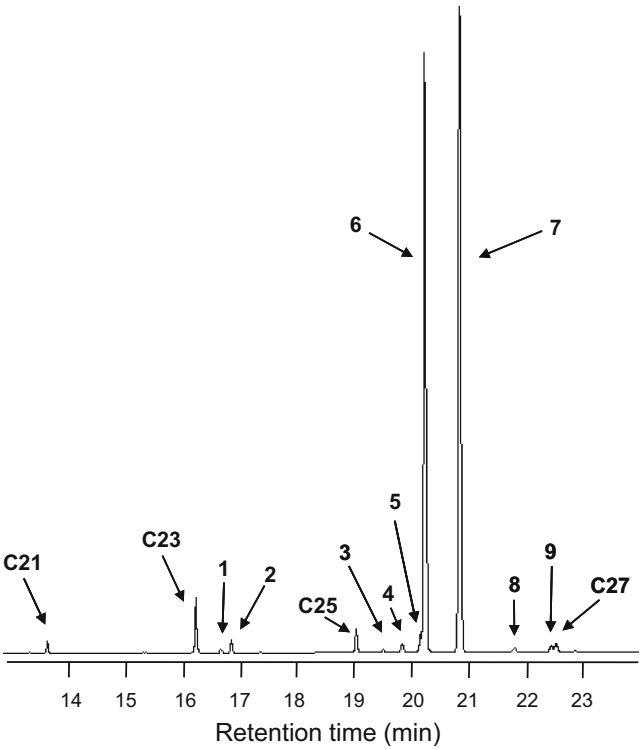

Fig. 1 Total ion chromatogram of GC-MS analysis of pheromone gland extract from female *Synanthedon haitangvora* on DB-Wax column. 1 (*Z*)-9-hexadecenyl acetate, 2 (*Z*)-11-hexadecenyl acetate, 3 (*Z*)-9-octadecenyl acetate, 4 (*Z*)-13-octadecenyl acetate, 5 (*E*,*Z*)-3,13-octadecadienyl acetate, 6 (*Z*,*Z*)-3,13-octadecadienyl acetate, 7 (*E*,*Z*)-2,13-octadecadienyl acetate, 8 (*Z*,*Z*)-3,13-octadecadien-1-ol, 9 (*E*,*Z*)-2,13-octadecadien-1-ol, C21 heneicosane, C23 tricosane, C25 pentacosane, C27 heptacosane

The mass spectra of peaks 5, 6, and 7 were similar and had diagnostic ions at *m/z* 308 (M^+), 248 (M^+-CH_3COOH), 219, 205, 191, and 61. This suggested that all three compounds were octadecadienyl acetates. Comparison of RIs of the natural compounds with the 3,13 and 2,13 isomers of octadecadienyl acetates on both columns confirmed that compounds 5, 6, and 7 were *E*3,*Z*13-18:OAc, *Z*3,*Z*13-18:OAc, and *E*2,*Z*13-18:OAc, respectively (Table 1). The mass spectrum of peaks 8 and 9 showed diagnostic fragment ions of octadecadien-1-ol at *m/z* 266 (M^+), 248 (loss of H_2O), 219, 205, and 191. The calculated RIs of peak 8 and the *Z*3,*Z*13-18:OH standard were the same on the DB-Wax and DB-23 columns (RIs of 2,669 and 2,673, respectively), and peak 9 coincided with *E*2,*Z*13-18:OH in GC retention times on both columns (RIs of 2,694 and 2,687, respectively). The amount of the major component *E*2,*Z*13-18:OAc present in the gland extract was estimated as 21.5 ng per female (N=15), and the relative ratio of compounds 1–9 in gland extracts was 0.2:1.5:0.4:3:2:87:100:1:1.5.

Field Experiments A total of 37 *S. haitangvora* males were trapped in field experiment 1, with no catch in control traps. *Z*3,*Z*13-18:OAc as a single component was attractive to males, while *E*2,*Z*13-18:OAc was unattractive alone (Fig. 2). Traps baited with a two-component blend of *Z*3,*Z*13-18:OAc and *E*2,*Z*13-18:OAc in a 1:1 ratio enhanced catches compared with traps baited with *Z*3,*Z*13-18:OAc alone or other blend ratios of the two components. In a subsequent experiment, a total of 394 moths were caught during the 4 days. Addition of *Z*3,*Z*13-18:OH to the binary blend of *Z*3,*Z*13-18:OAc and *E*2,*Z*13-18:OAc strongly inhibited attraction of *S. haitangvora* males (Fig. 3). Minor components identified in gland extracts showed no synergistic effect. Moreover, the full nine-component blend did not increase trap catches compared with traps baited with the primary binary blend. A total of 98 moths were captured in the final experiment that tested different doses of pheromone. Increasing loadings of the optimum pheromone blend in the lures from 0.1 mg to 2.0 mg resulted in increasing attractiveness of the lure (Fig. 4). There was a strong positive correlation between dose and catch, within the range of exposures tested (r^2=0.91, P<0.01).

Table 1 Retention indices of octadecadienyl compounds (peaks 5, 6, 7, 8 and 9) present in gland extracts of female *Synanthedon haitangvora* and synthetic 3,13- and 2,13-octadecadienyl compounds on DB-Wax and DB-23 columns

Compound	DB-Wax	DB-23
Female extract		
Peak 5	2,576	2,620
Peak 6	2,582	2,633
Peak 7	2,616	2,653
Peak 8	2,669	2,673
Peak 9	2,694	2,687
Authentic standard		
(*E*,*E*)-3,13-octadecadienyl acetate	2,568	2,594
(*E*,*Z*)-3,13-octadecadienyl acetate	2,576	2,620
(*Z*,*E*)-3,13-octadecadienyl acetate	2,574	2,613
(*Z*,*Z*)-3,13-octadecadienyl acetate	2,582	2,633
(*E*,*E*)-2,13-octadecadienyl acetate	2,608	2,627
(*E*,*Z*)-2,13-octadecadienyl acetate	2,616	2,653
(*Z*,*E*)-2,13-octadecadienyl acetate	2,582	2,613
(*Z*,*Z*)-2,13-octadecadienyl acetate	2,589	2,633
(*E*,*E*)-3,13-octadecadien-1-ol	2,633	2,612
(*E*,*Z*)-3,13-octadecadien-1-ol	2,641	2,632
(*Z*,*E*)-3,13-octadecadien-1-ol	2,661	2,646
(*Z*,*Z*)-3,13-octadecadien-1-ol	2,669	2,673
(*E*,*E*)-2,13-octadecadien-1-ol	2,692	2,659
(*E*,*Z*)-2,13-octadecadien-1-ol	2,694	2,687
(*Z*,*E*)-2,13-octadecadien-1-ol	2,689	2,681
(*Z*,*Z*)-2,13-octadecadien-1-ol	2,694	2,705

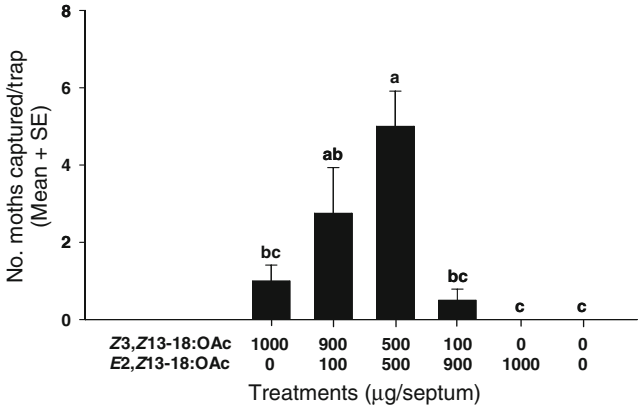

Fig. 2 Number of *Synanthedon haitangvora* males captured in traps baited with lures containing different ratios of *Z*3,*Z*13-18:OAc and *E*2,*Z*13-18:OAc at apple orchards in Suwon, Korea, 27–30 August 2008 (N=4). *Bars* with the same letter are not significantly different (Tukey's test: $P>0.05$)

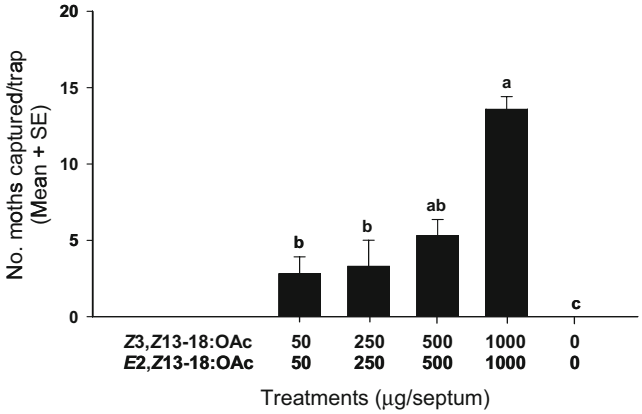

Fig. 4 Number of *Synanthedon haitangvora* males captured in traps baited with different doses of *Z*3,*Z*13-18:OAc and *E*2,*Z*13-18:OAc at apple orchards in Suwon, Korea, 7–10 September 2008 (N=4). *Bars* with the same letter are not significantly different (Tukey's test: $P>0.05$)

Discussion

GC-MS analyses of female *S. haitangvora* pheromone gland extract showed that two major octadecadienyl acetates were present at an approximately 1:1 ratio. To determine the double-bond positions of these diene acetates, dimethyl disulfide (DMDS) derivatization of the female extracts was performed as described by Vincenti et al. (1987). However, as in the study by Naka et al. (2006), the mass spectrum of the DMDS adducts did not show diagnostic ions indicating the double-bond positions of the dienes. In most of the known cases, moths in the family Sesiidae utilize octadecadienyl compounds with 3,13 or 2,13 positions of unsaturation as major components of their female sex pheromones (El-Sayed 2009). Based on this information, the two major compounds, *Z*3,*Z*13-18:OAc and *E*2,*Z*13-18:OAc, were identified by comparison of retention indices on two GC columns with authentic standards of all four isomers of the 3,13- and 2,13-octadecadienyl acetates.

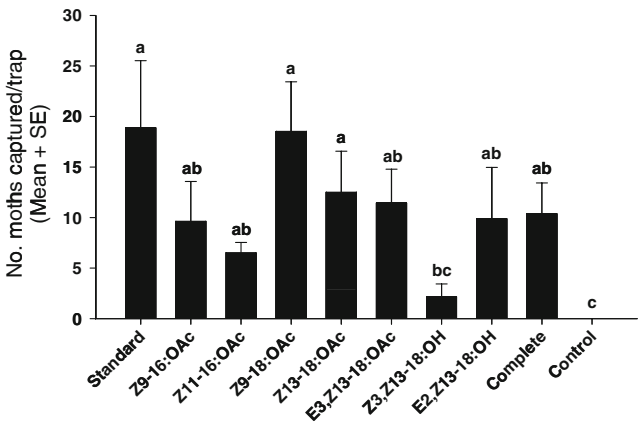

Fig. 3 The effect of adding 2% of different minor components to standard baits with 1 mg/septum of *Z*3,*Z*13-18:OAc and *E*2,*Z*13-18:OAc on captures of male *Synanthedon haitangvora* at apple orchards in Suwon, Korea, 2–5 September 2008 (N=4). The complete blend consisted of nine components in a ratio found in the pheromone gland extract. *Bars* with the same letter are not significantly different (Tukey's test: $P>0.05$)

Field trapping experiments showed that *Z*3,*Z*13-18:OAc and *E*2,*Z*13-18:OAc were necessary for optimal attraction of male *S. haitangvora*, and males were preferentially attracted to the blend found in gland extracts. Conversely, none of the other geometrical isomers of major components were attractive to male moths (data not shown). During the course of this study, no other sesiid species were attracted to a 1:1 mixture of *Z*3,*Z*13-18:OAc and *E*2,*Z*13-18:OAc. Thus, we suggest that this blend is a specific and effective attractant for monitoring *S. haitangvora*.

*Z*3,*Z*13-18:OAc and *E*2,*Z*13-18:OAc have been reported as either sex pheromone components or attractants in many species of Sesiidae (El-Sayed 2009). Like *S. haitangvora*, *Synanthedon scitula* and *Melittia cucurbitae* utilize mixtures of *Z*3,*Z*13-18:OAc and *E*2,*Z*13-18:OAc in their sex pheromones (Klun et al. 1990; Zhang et al. 2005). *Z*3,*Z*13-18:OAc is the major component of the pheromone blend of *S. scitula*, whereas *E*2,*Z*13-18:OAc constitutes the major component for *M. cucurbitae*. Therefore, species-specific pheromone signals by using different ratios might play a role in reproductive isolation if *S. haitangvora* is sympatric with *S. scitula* and *M. cucurbitae* in other areas of the world.

The effects of minor glandular components on field trap catches varied. The addition of *Z*3,*Z*13-18:OH to the binary blend of *Z*3,*Z*13-18:OAc and *E*2,*Z*13-18:OAc significantly decreased attraction for *S. haitangvora* males. This result suggests that *S. haitangvora* males may be not attracted to *Nokona pernix* and *Paranthrene tabaniformis* females that

emit *Z*3,*Z*13-18:OH as their pheromone (Naka et al. 2006; Mozûraitis and Karalius 2007). On the other hand, addition of *E*3,*Z*13-18:OAc did not affect male attraction of *S. haitangvora* males to the standard bait. In field screenings of attractants for *Synanthedon tenuis*, Tamaki et al. (1977) confirmed an antagonistic effect of *E*3,*Z*13-18:OAc on male attraction when it was mixed with the major component, *Z*3,*Z*13-18:OAc, in a very low ratio. Therefore, *E*3, *Z*13-18:OAc may prevent cross-attraction between *S. haitangvora* and *S. tenuis* where they occur sympatrically in Korea and China. However, it is not known whether *Z*3, *Z*13-18:OH and *E*3,*Z*13-18:OAc are actually released from the female glands during calling. Further research is required to compare the attractiveness of pheromone blends actually emitted with those found in female gland extracts.

In addition, four monounsaturated acetates, *Z*9-16:OAc, *Z*11-16:OAc, *Z*9-18:OAc, and *Z*13-18:OAc, were identified as minor components of female *S. haitangvora* gland extracts, but the behavioral roles of these components remain unclear. Addition of monounsaturated acetates to two major components in biologically relevant rates had no significant effect on attraction of conspecific males in the present study. Previous studies with other sesiid species have shown that monounsaturated acetates are present in female pheromone glands, but no biological activity was observed in the field trials (Klun et al. 1990; Mozûraitis et al. 2006). These results suggest that monounsaturated acetates found in gland extracts from sesiid moths may just be by-products of the biosynthesis of octadecadienyl acetates. Further studies are necessary to determine the biosynthetic pathway for producing the sex pheromone components in sesiid moths.

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walking up the tree bole. They suggested that the female initiates courtship by walking close to a male on the upper branches of the canopy. The male follows the female, tapping her body with the forelegs and the antennae. This description by Morgan and Stewart (1966) strongly suggested the presence of a contact signal on the female cuticle.

In a preliminary study in the laboratory, *Sirex noctilio* males, when presented with freeze-killed females, responded with abdominal bending but did not do so after the cuticular coating of the dead females was removed by dipping the female in hexane (D.J.C., unpublished data). These preliminary results indicated the relevance of female cuticular compounds in the mating behavior. This is further supported by the work of Crook et al. (2008), who reported a high abundance of contact chemoreceptors on the antennae of *S. noctilio*.

Cuticular lipids are key compounds involved in the recognition of mates, caste, and kin, in a number of insect species (Howard 1993; Wyatt 2003). Hydrocarbons of the epicuticle, in particular, have been described as recognition cues, kairomones, chemical mimicry agents, and sex pheromone components throughout the insect taxa (Blomquist et al. 1993; Howard and Blomquist 2005). While semiochemicals of hymenopterans in the sub order Apocrita frequently are reported, we know much less about the chemical ecology of insects in the other sub order, Symphyta, in which the family Siricidae belongs (Ayasse et al. 2001; Keeling et al. 2004). The primary focus of this study was to analyze the cuticular lipid coating of male and female *S. noctilio*, and to identify the contact pheromone that induces males to attempt copulation with females.

Methods and Materials

Insects Adults were reared out of Scots pine logs harvested near Syracuse, NY, USA. The first batch of infested trees were taken down in December 2007 and cut into approximately 60 cm-long logs. The logs were kept in barrels with a mesh lid at 25°C at the USDA PPQ rearing facility in Syracuse. Emergence of males started at the end of February 2008, followed thereafter by the emergence of females in March. A second batch of logs was taken into the rearing facility in May 2008. Emergence started in July and lasted until the beginning of August. Barrels were checked twice a week, and emerging insects were identified and separated by sex. Males and females were kept individually in screw cap glass jars, with a piece of paper towel inside, until used.

Solvent Dipping For the analysis of cuticular lipids, insects were freeze killed at age 1–6 days. On the day of analysis, bodies were thawed for 30 min at room temperature. Each wasp was placed individually into a 10 ml screw cap vial and extracted × 3, for 2 min, with 3 ml of hexane (Burdick & Jackson, 95%, Morristown, NJ, USA). The extracts were combined in a separate 10-ml screw cap vial. Solutions were held at 4°C until subsequent fractionation or analysis. For analysis, the body wash was evaporated under a gentle stream of nitrogen. The residue was redissolved in 300 µl of a 50 ng/µl solution of 16-methyl hexatriacontane in hexane.

For the bioassay experiments, 1 to 6-d-old females were freeze killed the night before the experiment. The bodies were thawed for 30 min at room temperature. Extraction was performed as described above. The extracts were combined in a 10-ml screw cap vial, and the body wash was evaporated under a gentle stream of nitrogen. Samples were redissolved with the appropriate amount of hexane to give body wash solutions of 1/3, 1, or 3 female equivalents (FE) per 100 µl. Washed females were dried in a fume hood for 30 min before application of any treatment.

Analysis of the Cuticular Washes Amounts of compounds were quantified with an Agilent 6890 gas chromatograph-flame ionization detector (GC-FID) system equipped with an Equity-5 column (30 m×0.2 mm×0.2 µm; Supelco, Bellefonte, PA, USA). Quantification of compounds was based on their peak area values obtained from the data acquisition and analysis software (Chemstation D.01.00, Agilent). The output values were corrected with the relative response factors to calculate percentage composition and amounts of the identified compounds (Böröczky et al. 2008). For identification, selected samples were injected into an Agilent 6890N GC, equipped with an identical Equity-5 column, coupled with a 5973N Mass Selective Detector (MSD) in EI mode (+70 eV). For all the analyses, the oven temperature program was initially 50°C (held for 1 min) then programmed to 210°C at 20°C min^{-1}, then to 320°C at 3°C min^{-1} (and held for 25 min). The injector temperature was 280°C, in both systems, and the FID (in the GC) and transfer line (in the GC-MS) temperatures were 300°C. Samples were injected splitless (purge set at 0.75 min), and the carrier gas was helium at an average linear flow velocity of 25 cm s^{-1}.

Identification of the compounds was based primarily on their EI mass spectra (NIST05, Masslib) and their Kovats indices on the Equity-5 column (Van Den Dool and Kratz 1963; Kovats 1965). Position of the methyl branching of mono-, and dimethylalkanes was determined by using characteristic even- and odd-mass fragments of their respective mass spectra (Nelson, 2001) as well as by calculated retention indices (Carlson et al., 1998). Position of double bonds was determined by examining the mass spectral fragmentation of the dimethyl disulfide (DMDS)

adducts of the alkenes (Francis and Veland 1981; Vincenti et al. 1987). Identification of wax esters is tentative based on their molecular weight and the weight of the $[RCO\text{-}1]^+$ and $RCOOH_2^+$ fragments from their mass spectra. High resolution MS (HR-MS) analysis of the natural and synthetic compounds was performed at the Proteomics and Mass Spectrometry Core Facility at The Huck Institutes of the Life Sciences of The Pennsylvania State University.

Contact Behavioral Assay Behavioral experiments were performed between March 20 and April 18, and between July 16 and August 1, 2008. Live males used in the assays were 1-6-d-old. Treatments were prepared fresh each morning. Dead females (unwashed and hexane washed) were mounted, at the thorax, on a piece of wire. Treatment solutions were applied to previously washed dead females with a 10 µl syringe, thus ensuring that the solution covered the bodies evenly. Treated females were placed in a fume hood for 10 min to allow evaporation of the solvent. In the bioassay, the responses of males were tested by presenting a female 1–2 cm away from the males. The typical sequence of responses to an active treatment was, walking up to the treated dead female, antennation of the abdomen and/or grabbing the dead female with the forelegs, mounting, and either abdominal bending or a copulation attempt. Each observation lasted 20–25 sec or was terminated earlier if the male attempted copulation or flew away. We counted copulation attempts as being a positive response. Each morning a new group of males was tested with an unwashed dead female (UDF), and only the positively responding males were used on that day in the bioassay (6–12 males per day). Each set of treatments was replicated 17–24 times over 3–4 d. The order in which the treatments were presented varied each day. A treatment was presented to all the males before switching to the next treatment, with males allowed to rest half an hour between treatments. Each set of treatments included a negative and a positive control. The negative control (C) was prepared by evaporating 9 ml hexane and redissolving the residue in 100 µl of hexane. For the positive control, an unwashed dead female (UDF) or a female with reapplied whole body wash (WB) was used. The sets of treatments tested were the following: a) Whole body wash reapplied at three concentrations: 1/3, 1, and 3FE (WB1/3, WB, and WB3, respectively); and the controls C and UDF. b) Fractions of the female body wash at 1FE: alkanes (A), monoalkenes (MA), dialkenes (DA), and more polar lipids (P) (see below); and the controls C and WB. c) Synthesized components of MA at 1FE: (*Z*)-7-heptacosene (Z7C27), (*Z*)-9-heptacosene (Z9C27), (*Z*)-7-nonacosene (Z7C29), and (*Z*)-9-nonacosene (Z9C29) individually (see below), a mix of all four (Mix4), MA; and the controls C and UDF. d) Binary combinations of the three most active components at 1FE: Z7C27+Z7C29, Z7C27+Z9C29, and Z7C29+Z9C29, a mix of the three components (Mix3), Mix4, MA; and the controls C and UDF.

For each treatment within a set, the significance of difference from the positive control was determined by using the 2-tail values of the Fisher's exact test.

Fractionation of the Female Body Wash Body washes of ten females were combined and evaporated to dryness, which resulted in approximately 4 mg solid material. This was redissolved in 50 µl of hexane and loaded onto a small flash chromatography column, consisting of a Pasteur pipette, with a ball of glass wool as a plug and approximately 300 mg of silica gel (Sigma-Aldrich, 230–400 mesh, 60 Å). Compounds were eluted with hexane in two 1-ml fractions, followed by three 1-ml fractions of diethyl-ether. The hexane fractions were combined to provide a hydrocarbon mix, while the combined diethyl-ether fractions gave a mixture of more polar lipids. The latter was evaporated to dryness and redissolved in 1 ml of hexane resulting in a 1FE/100 µl solution (P).

The hydrocarbon fraction was further separated on 300 mg of silver nitrate-treated silica (Li et al. 1995). The fraction, in hexane, was loaded onto the column, and the alkanes were eluted with hexane (two 1-ml fractions), the monoalkenes with dichloromethane (B&J, High Purity Solvent; five 1-ml fractions), and the dialkenes with acetonitrile (EMD, Omnisolv, High Purity Solvent; two 1-ml fractions). Fractions containing compounds of the same class were combined and evaporated to dryness. Residues were redissolved in 1 ml of hexane, resulting in the following 1FE /100 µl solutions: alkanes (A), monoalkenes (MA), and dialkenes (DA).

Syntheses Z9C27 was prepared as the main geometric isomer from the Wittig reaction of nonyltriphenyl phosphonium bromide and octadecanal. A significant amount of the (*E*)-isomer was formed by this route and required additional chromatography for purification. Partial hydrogenation of the appropriate alkynes was used to provide alkenes Z7C27, Z7C29, and Z9C29. The corresponding alkanes were produced in 3–5% as a result of over-reduction, but they were easily removed by flash chromatography. The alkynes were conveniently prepared by condensing the appropriate alkyl iodides with either 1-decynyl lithium or 1-octynyl lithium in the presence of 1, 3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone (DMPU) (Bengtsson and Liljefors 1988). The identity of the compounds was confirmed by their mass spectra and the exact mass of the molecular ion determined by HR-MS. Melting points are uncorrected.

Preparation of 7-Heptacosyne A solution containing 0.33 g (2 mmol) of distilled 1-octyne in 10 ml of tetrahydrofuran

(THF) was cooled to −70°C and treated with 2 ml of 1.6 M *n*-BuLi. After 45 min, a solution containing 0.39 g (1 mmol) of 1-iodononadecane (Marukawa et al., 2001) in 3 ml of DMPU was added dropwise, and the mixture was allowed to warm to room temperature overnight. The mixture was treated with 5 ml of saturated NH_4Cl and taken up in 70 ml of petroleum ether. The organic layer was washed × 3 with 10 ml of water, and dried over anhydrous $MgSO_4$. After filtration, the solvent was removed in vacuum, and the residue was purified by flash chromatography (hexane/silica gel) to provide 0.19 g of 7-heptacosyne as a waxy solid: mp 35–37°C, EI-MS *m/z* 376 [M^+](2), 292(1), 291(2), 278(2), 277(1), 250(1), 222(1), 208(1), 207(2), 194(1), 180(2), 179(2), 166(10), 165(10), 152(5), 151(7), 138(9), 137(11), 124(15), 123(24), 111(15), 110(30), 109(58), 97(37), 96(82), 95(88), 83(48), 82(83), 81(100), 80(12), 79(16), 71(14), 69(40), 68(30), 67(73), 57(41), 55(56), 54(32), 43(57), 41(39). HR-MS *m/z* 376.4080; calculated for $C_{27}H_{52}$, 376.4069.

Preparation of 7-Nonacosyne Coupling 1-octyne and 1-iodoheneicosane (Mori and Wu, 1992), as described for 7-heptacosyne, provided 0.18 g of 7-nonacosyne as a waxy solid: mp 41–43°C, EI-MS *m/z* 404 [M^+](2), 320(1), 319 (2), 306(2), 305(1), 207(1), 180(3), 179(2), 166(11), 165 (11), 152(6), 151(7), 138(11), 137(12), 124(17), 123(25), 111(18), 110(33), 109(60), 97(41), 96(90), 95(89), 85(10), 83(51), 82(87), 81(100), 80(12), 79(15), 71(18), 69(44), 68(32), 67(74), 57(45), 55(56), 54(30), 43(59), 41(36). HR-MS *m/z* 404.4369; calculated for $C_{29}H_{56}$, 404.4382.

Preparation of 9-Nonacosyne Coupling 1-decyne and 1-iodononadecane (Marukawa et al., 2001), as described for 7-heptacosyne, provided 0.3 g of 9-nonacosyne as a waxy solid: mp 35–37°C, EI-MS *m/z* 404 [M^+](2), 347(1), 278 (2), 277(1), 264(1), 222(1), 208(2), 207(2), 195(1), 194(5), 193(5), 152(5), 151(6), 138(14), 137(22), 124(23), 123(26), 111(16), 110(20), 109(38), 97(40), 96(74), 95(72), 83(49), 82(93), 81(100), 80(12), 79(15), 71(15), 69(38), 68(25), 67(64), 57(46), 56(10), 55(58), 54(26), 43(52), 41(33). HR-MS *m/z* 404.4382; calculated for $C_{29}H_{56}$, 404.4382.

Preparation of Z7C27 A solution containing 100 mg of 7-heptacosyne, 2 drops of quinoline, and 75 mg of Lindlar catalyst (Aldrich Chemical Co.), in 25 ml of ethanol and 2 ml of ethyl acetate, was cooled to 10°C and hydrogenated at atmospheric pressure. The reaction products were followed by GC-MS analysis; this indicated the absence of starting alkyne after 3 h. The mixture was filtered through celite, and after the solvent was removed *in vacuo*, flash chromatography (hexane/silica gel) provided 85 mg of Z7C27, EI-MS *m/z* 378 [M^+](5), 237(1), 223(1), 222(1), 210(1), 209(2), 208(1), 196(1), 195(2), 194(1), 182(2), 181(3), 180(2), 167(6), 153(9), 139(15), 125(30), 112(12), 111(54), 110(10), 99(11), 98(17), 97(91), 96(22), 85(37), 84(27), 83(88), 82(29), 81(15), 71(50), 70(35), 69(68), 68(13), 67(17), 57(100), 56(39), 55(81), 54(14), 43(68), 41(39). HR-MS *m/z* 378.4232; calculated for $C_{27}H_{54}$, 378.4226.

Preparation of Z7C29 Hydrogenation of 7-nonacosyne, as described above, provided Z7C29, EI-MS *m/z* 406 [M^+](4), 251(1), 250(1), 237(1), 236(1), 224(1), 223(2), 222(1), 210(1), 209(2), 208(1), 196(1), 195(3), 194(1), 183(1), 182(2), 181(4), 180(2), 167(6), 153(11), 139(18), 125(34), 112(12), 111(58), 110(10), 99(13), 98(18), 97(96), 96(25), 95(10), 85(39), 84(26), 83(90), 82(30), 81(16), 71(54), 70(35), 69(69), 68(15), 67(20), 57(100), 56(34), 55(71), 54(13), 43(74), 41(37). HR-MS *m/z* 406.4522, calculated for $C_{29}H_{58}$, 406.4539.

Preparation of Z9C29 Hydrogenation of 9-nonacosyne, as described above, provided Z9C29, EI-MS *m/z* 406 [M^+](4), 251(1), 250(1), 237(1), 236(1), 224(1), 223(2), 222(1), 210(1), 209(2), 208(1), 196(1), 195(3), 194(1), 183(1), 182(2), 181(4), 180(2), 167(6), 153(11), 139(18), 125(34), 112(12), 111(58), 110(10), 99(13), 98(18), 97(96), 96(25), 95(10), 85(39), 84(26), 83(90), 82(30), 81(16), 71(54), 70(35), 69(69), 68(15), 67(20), 57(100), 56(34), 55(71), 54(13), 43(74), 41(37). HR-MS *m/z* 406.4544; calculated for $C_{29}H_{58}$, 406.4539.

After purification, compounds Z7C27, Z7C29, and Z9C29 were at least 99% pure (*Z*)-isomers based on the peak area values by GC.

Preparation of Z9C27 A suspension of 0.86 g (2 mmol) of nonyltriphenylphosphonium bromide in 10 ml THF was cooled to −10°C under argon and treated with 1.5 ml of 1.6 M butyl lithium in hexanes to provide an orange mixture. After 30 min, a solution containing 0.54 g of octadecanal in 5 ml THF was added dropwise, and the mixture was stirred overnight and allowed to warm to room temperature. The mixture was partitioned between diethyl ether and aqueous NH_4Cl. The ether layer was dried over anhydrous $MgSO_4$, filtered, and the solvent removed *in vacuo*. The residue was taken up in petroleum ether and filtered through celite, and the fitrate concentrated *in vacuo*. Flash chromatography (hexane/silica gel) provided 200 mg of a 5:1 mixture of (*Z*)- and (*E*)-isomers; EI-MS *m/z* 378 [M^+](2), 237(1), 210(1), 209(2), 208(1), 196(1), 195(2), 194(1), 182(2), 181(3), 180(2), 167(6), 153(9), 139(15), 125(30), 112(12), 111(54), 110(10), 99(11), 98(17), 97(91), 96(22), 85(37), 84(27), 83(88), 82(29), 81(15), 71(50), 70(35), 69(68), 68(13), 67(17), 57(100), 56(39), 55(81), 54(14), 43(68), 41(39). HR-MS *m/z* 378.4232, calculated for $C_{27}H_{54}$, 378.4226. A small portion (4 mg) of the 5:1 (*Z*)/(*E*)-mix was purified further on a silver nitrate treated silica

column (400 mg) by sequential elution with hexane, 5% dichloromethane in hexane, 10% dichloromethane in hexane, and 50% dichloromethane in hexane, all in 1-ml fractions. Fractions 2–6, in 10% dichloromethane in hexane, and fractions 1–3, in 50% dichloromethane in hexane, were combined, which resulted in 3 mg of Z9C27, 98% pure (*Z*)-isomer based on the peak area values by GC.

Results

Cuticular Lipids of S. noctilio The hexane-extractable cuticular lipids of *S. noctilio* consisted predominantly of alkanes (C21-C33), mono- and dimethyl alkanes (C23-C37), mono- and dialkenes (C23-C31), and wax esters (C36-C44). (Fig. 1, Table 1). The hydrocarbon components of the cuticle were sexually dimorphic. While *n*-alkenes were one of the major groups of the hexane body wash of females (29% in females, 4% in males), methyl-branched alkanes were more abundant in the male body wash (28% in males, 2% in females). Additonally, a few lower molecular-weight (C23-C25) monoalkenes were present on the male cuticle. Wax esters and *n*-alkanes were abundant in both sexes (37% and 31% in males, and 38% and 31% in females, respectively).

Activity-Guided Bioassay UDF and WB positive controls, respectively, elicited copulation attempts in 65% and 60% of males (not significantly different, Fisher's exact test). However, WB controls at one-third the amount (1/3WB) elicited a significantly weaker response from males than when the same treatment was tested at 1 FE (Fig. 2a). Negative C controls did not elicit copulation attempts in any of the males tested across all experiments (Fig. 2), thus demonstrating that hexane washing removed the cuticular signals.

The A fraction did not elicit a positive response in any of the males (N=17) tested, whereas a weak response was observed to the DA and more polar P fractions. The response to the MA fraction was not different statistically

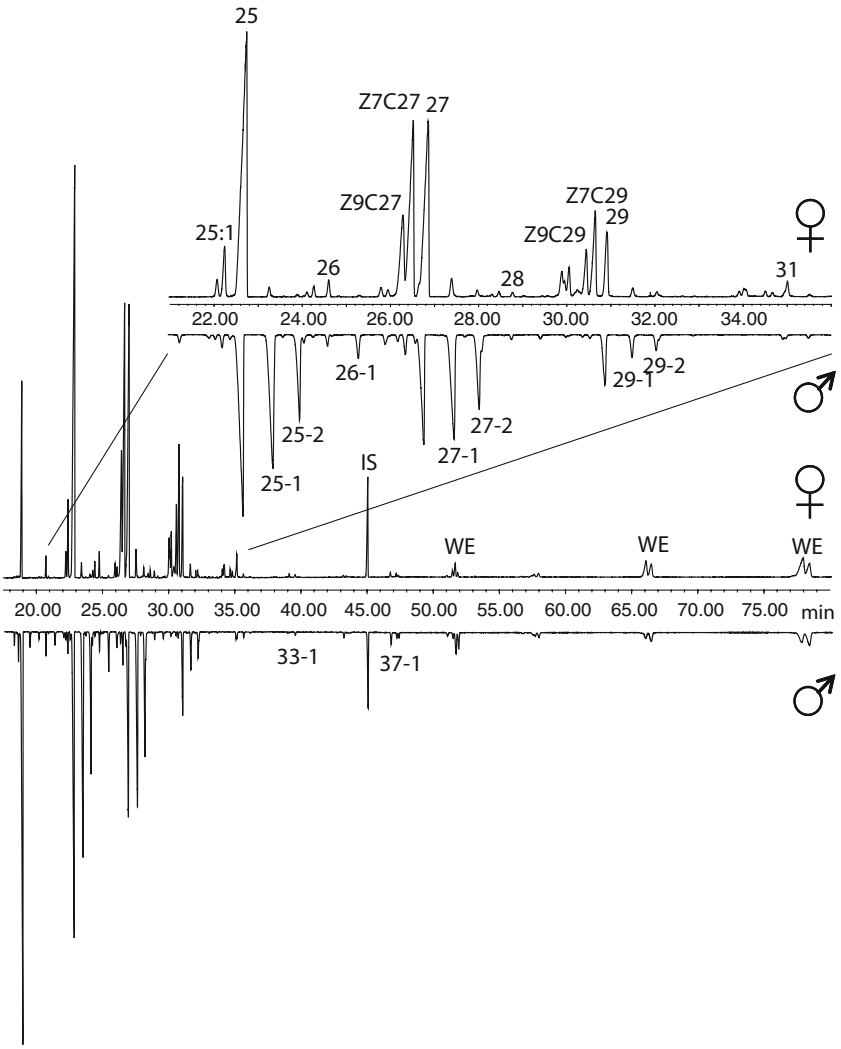

Fig. 1 Representative gas chromatograms of the whole body wash of female (*top*) and male (*bottom*) *Sirex noctilio*. Each sample is a hexane extract of one individual with 50 ng/μl 16-methyl hexatriacontane as internal standard. Coding is the same as in Table 1, for example, pentacosane (25), pentacosene (25:1), (*Z*)-9-nonacosene (Z9C29), methyl heptacosane (27-1), dimethyl pentacosane (25-2), and wax ester (WE), etc.

Table 1 Cuticular lipids of the male and female *S. noctilio*

Code	Compound[a,b]	RI[c]	Amount (μg/wasp)[d,e]			
			Males		Females	
			Mean	S.E.M.	Mean	S.E.M.
21	Heneicosane	2100	0.24	0.07	0.13	0.03
22	Docosane	2200	0.19	0.05	0.12	0.03
23:1	?-Tricosene	2279	0.12	0.09	tr	
U	Unknown	2284	0.54	0.27	0.18	0.06
23	Tricosane	2300	21.68	5.76	11.98	2.47
23-1	11-/9-Methyl tricosane	2332	0.43	0.14	tr	
	7-Methyl tricosane	2336	tr		nd	
23-2	9,13-Dimethyl tricosane	2366	tr		nd	
	7,11-Dimethyl tricosane	2370	0.15	0.06	tr	
24	Tetracosane	2400	0.72	0.16	0.76	0.09
24-1	12-/11-/10-/9-/8-Methyl tetracosane	2433	0.41	0.14	nd	
24-2	8,12-Dimethyl tetracosane	2466	0.23	0.08	nd	
25:1	9-Pentacosene	2473	0.59	0.18	1.07	0.25
	7-Pentacosene	2481	2.10	1.20	3.48	0.82
25	Pentacosane	2500	20.96	4.67	33.43	7.71
25-1	13-/11-/9-Methyl pentacosane	2538	13.86	3.99	0.49	0.12
U	Unknown	2549	0.12	0.04	nd	
25-2	9,13-Dimethyl pentacosane	2568	4.63	2.17	0.10	0.04
	7,13-Dimethyl pentacosane	2573	0.35	0.18	nd	0.00
26:1	9-Hexacosene	2576	nd		0.33	0.08
	7-Hexacosene	2583	nd		0.69	0.17
25-2	5,15-/5,17-Dimethyl pentacosane?	2583	0.22	0.06	nd	
26	Hexacosane	2600	0.75	0.17	1.07	0.18
26-1	13-/12-/11-/10-/9-/8-Methyl hexacosane	2633	1.65	0.49	tr	
27:2	8,18-/?-Heptacosadiene	2657	nd		0.72	0.16
26-2	10,14-/11,15-Dimethyl hexacosane	2662	0.62	0.30	nd	
27:2	7,19-Heptacosadiene	2664	nd		0.56	0.11
27:1	*(Z)-9-Heptacosene, Z9C27*	*2676*	*1.19*	*0.45*	*11.71*	*2.91*
	(Z)-7-Heptacosene, Z7C27	*2684*	*3.51*	*1.79*	*27.54*	*6.67*
27	Heptacosane	2700	13.03	2.91	23.94	5.96
27-1	13-/11-/9-Methyl heptacosane	2737	15.16	3.36	1.20	0.26
U	Unknown	2749	0.14	0.06	nd	
27-2	11,15-/9,13-Dimethyl heptacosane	2764	6.30	2.61	0.47	0.08
28:1	9-Octacosene	2777	nd		0.23	0.05
	7-Octacosene	2786	nd		0.40	0.09
28	Octacosane	2800	0.37	0.09	0.48	0.10
28-1	14-/13-/12-/11-Methyl octacosane	2831	0.36	0.09	nd	
29:2	9,19-Nonacosadiene	2854	nd		3.66	0.77
	7,17-Nonacosadiene	2859	nd		1.58	0.31
28-2	14,18-/11,15-Dimethyl octacosane?	2859	0.11	0.02	nd	
29:2	7,19-Nonacosadiene	2862	nd		4.03	0.85
	?-Nonacosadiene, 14-/13-/12-/11-/10-Nonacosene	2871	nd		1.86	0.40
29:1	*(Z)-9-Nonacosene, Z9C29*	*2878*	*0.40*	*0.13*	*5.52*	*1.10*
	(Z)-7-Nonacosene, Z7C29	*2886*	*1.07*	*0.70*	*8.44*	*1.82*
29	Nonacosane	2900	5.75	1.76	9.42	3.05

Table 1 (continued)

Code	Compound[a,b]	RI[c]	Amount (μg/wasp)[d,e]			
			Males		Females	
			Mean	S.E.M.	Mean	S.E.M.
29-1	15-/13-/11-Methyl nonacosane	2932	2.43	0.43	0.77	0.15
29-2	13,17-Dimethyl nonacosane	2958	1.42	0.55	0.57	0.11
30	Triacontane	3000	0.12	0.02	tr	
31:2	9,19-Untriacontadiene	3050	nd		0.82	0.11
	7,17-Untriacontadiene	3056	nd		1.03	0.09
	7,19-Untriacontadiene	3059	nd		0.91	0.20
	?-Untriacontadiene	3063	nd		0.17	0.04
	?-Untriacontadiene	3069	nd		0.15	0.04
31:1	?-Untriacontene	3072	nd		0.12	0.04
	9-Untriacontene	3081	nd		0.45	0.09
	7-Untriacontene	3088	nd		0.31	0.10
31	Untriacontane	3100	0.63	0.11	0.65	0.28
31-1	15-/13-Methyl untricontane, (Cholesterol)	3130	0.56	0.10	0.34	0.06
33	Tritriacontane	3300	0.14	0.01	0.14	0.03
33-1	13-Methyl tritriacontane, (Stigmasterol)	3333	0.36	0.05	0.28	0.07
35-1	17-/15-/13-Methyl pentatriacontane	3531	0.71	0.12	0.27	0.02
37-1	17-/15-/13-Methyl heptatriacontane	3715	1.93	0.27	0.84	0.08
WE	C17:1CO-OC18:0	3737	1.49	0.08	0.98	0.09
	C17:1CO-OC18:0	3745	0.98	0.24	0.52	0.05
	C17:1CO-OC20:0	3937	1.74	0.33	2.14	0.16
	C17:1CO-OC20:0	3947	8.44	2.17	7.62	1.34
	C17:1CO-OC20:0	3955	4.51	1.72	2.54	0.53
	C17:1CO-OC22:0	4136	2.87	0.89	3.06	1.15
	C17:1CO-OC22:0	4144	4.00	1.65	2.44	0.48
	C17:1CO-OC24:0	4348	12.15	4.23	13.85	2.40
	C17:1CO-OC24:0	4358	13.05	4.72	11.61	2.02
	C17:1CO-OC26:0	>4400	17.78	6.46	34.35	5.73
	C17:1CO-OC26:0	>4400	14.88	6.01	18.62	3.57

[a] Compounds in italics were synthesized and tested in a bioassay.

[b] Partial identification of wax esters (WE) is based on their MS spectra in EI mode.

[c] Kovats indices on the Equity-5 column (for references see Methods).

[d] All averages are means of four.

[e] 'nd': not detected, 'tr': <0.10 μg/wasp.

from the response to WB (Fig. 2b), hence, we investigated that fraction further. The major components of MA were identified as pentacosenes, heptacosenes, and nonacosenes based on their retention indices and mass spectra. The position of the double bond of the most abundant compounds was shown to be 7 and 9, based on the mass spectra of the DMDS adducts. Additional isomers in lower amounts were nonacosenes with the double bond being more toward the middle of the chain (Table 1). The four most abundant components of MA, 7- and 9-heptacosene, and 7- and 9-nonacosene, were synthesized, and the geometries of the double bonds in the natural compounds were determined by comparison of the retention times on the Equity-5 column. For all four monoalkenes, the natural compounds were the (*Z*)-isomers. Purification of the crude synthetic products by flash chromatography on silica and silver nitrate-treated silica led to 98–99% pure (*Z*)-isomers, which were tested in our bioassay at 1FE (for amounts see Table 1). One of the compounds, Z9C27, did not elicit significant responses from males (N=24), while the other

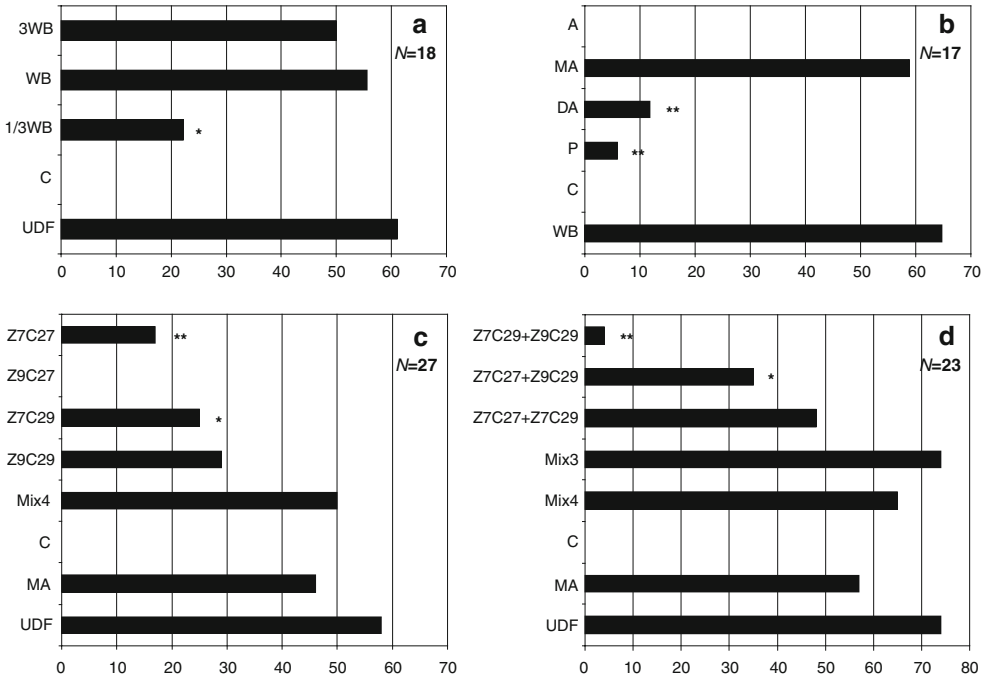

Fig. 2 Percentages of males that attempted copulation with dead females having various treatments applied in four sets of treatments. (**a**) Three concentrations of the female whole body wash: 3 female equivalents (FE) (3WB), one FE (WB), and one third FE (1/3WB). (**b**) Fractions of the body wash at one FE: alkane fraction (A), monoalkene fraction (MA), dialkene fraction (DA), and the fraction of more polar cuticular lipids (P). (**c**) Four synthetic components of MA: (*Z*)-7-heptacosene (Z7C27), (*Z*)-9-heptacosene (Z9C27), (*Z*)-7-nonacosene (Z7C29), and (*Z*)-9-nonacosene (Z9C29). (**d**) Binary combinations of the active synthesized monoalkenes. Positive control was either an unwashed dead female (UDF; **a**, **c**, and **d**) or whole body wash reapplied (WB; **b**). Negative control was hexane reapplied (C). Within each set the statistical significance of the difference of a treatment from the positive control was calculated using Fisher's exact test; *: $P<0.05$, **; $P<0.01$

three did (Fig. 2c). The mixture of the three active components at 1 FE (Mix3), and also the binary mixture of Z7C27 and Z7C29, elicited activity from males not different statistically from that elicited by UDF. The results of testing binary combinations of the three active monoalkenes indicated that Z7C27 was an essential component of the blend (Fig. 2d).

Discussion

We identified three major components, Z7C27, Z7C29, and Z9C29, of the cuticular lipids of female *S. noctilio* that elicited copulation attempts in conspecific males. The responses of males to a binary mixture of synthetic Z7C27 and Z7C29 or to a mixture of these three compounds were not different statistically from the responses to unwashed dead females, indicating that these compounds accounted for most, if not all, the cuticular activity. When Z7C27 was not present in the blend, the responses of males dropped significantly (Fig. 2d, $P<0.01$). Further work on these compounds should test more rigorously for possible additive and/or synergistic effects in mixtures of these compounds.

Cuticular monoalkenes are well-known to act as contact sex pheromone components and sex recognition compounds in some cerambicid beetle (Ginzel et al. 2003, 2006) and *Drosophila* species (Howard 1993; Howard and Blomquist 2005). These compounds are odd chain-length alkenes, ranging in chain length from 25 to 33 carbons, with (*Z*) geometry across the double bond. Among hymenopterans, various parasitic wasps are reported to use a mix of cuticular alkenes and, more often, alkadienes as volatile sex pheromones (Keeling et al. 2004). In the suborder Symphyta, the family Diprionidae (conifer sawflies) is the most extensively studied. Many of the species use female-produced volatile sex pheromones that are methyl-branched secondary alcohols and esters thereof (Keeling et al. 2004). However, no reports are available on the role of cuticular compounds in mating behavior. To our knowledge this is the first description of cuticular hydrocarbons that serve as contact sex pheromone components in this ancient hymenopteran suborder.

In our bioassays, 65% of males attempted copulation with UDF controls, although all males tested had responded positively during the selection process earlier in the day. That we did not observe 100% positive responses to our positive control treatment is likely due to some males being

affected by frequent handling. Also, defrosted females sometimes had their wings in a twisted position, which restricted the movements of the male; males can copulate with a live female only after pressing down the wings of the female tight to her body (unpublished observation). Moreover, we only counted attempted copulation, the clearest behavioral response, as a positive response for our observations, and we did not include other positive responses, such as agitated wing-flapping and abdominal bending. Other variation observed in responses of males to UDF across treatment sets is likely due to differences in age (1-6-d-old males were used), changes in barometric pressure, relative humidity, temperature, and light conditions.

Our laboratory bioassay tested only contact cuticular chemicals for activity in mating. It seems likely that other factors (olfactory, visual, tactile, and auditory) may provide additional cues to males during mate location and selection. To date no evidence has been found for the existence of a long-range sex pheromone component in the Siricidae. Some hymenopterans are hill-topping insects and use rendezvous sites to find their mate (Alcock and Dodson 2008). Similar behavior has not been observed with *S. noctilio*. Although females may fly into male swarms, there are no behavioral data published that investigate the cause of such behavior; it is difficult to determine in a laboratory bioassay what type of cue (olfactory, visual, and/or auditory) may be used in such behavior. After emergence, both sexes are positively phototactic (Morgan and Stewart 1966), and it is likely that both sexes are attracted by host volatiles to the same area since their antennae are responsive to volatile terpenes produced by pines (Simpson 1976). Indeed, females can be caught using girdled pine trees as lures; however, males are not found in the same traps that are usually placed way under the canopy. Thus, it remains to be investigated how the two sexes locate each other.

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behavioral steps performed mainly by males (Rojas et al. 1990; Manrique and Lazzari 1994; Pires et al. 2004; Vitta and Lorenzo 2009). In addition, female receptivity to male copulatory attempts has been shown to affect mating success in *Triatoma infestans*, *Panstrongylus megistus*, and *T. brasiliensis* (Manrique and Lazzari 1994; Pires et al. 2004; Vitta and Lorenzo 2009). Few studies have been conducted on the effect of chemical cues on mating behavior in triatomines. Electrophysiological studies have shown that copulating pairs of *T. infestans* release odors that elicit responses from the antennae of males (De Brito Sánchez et al. 1995). Several authors have suggested that chemical signals are released by copulating pairs, leading to the aggregation of surrounding males (Baldwin et al. 1971; Manrique and Lazzari 1995).

Triatoma brasiliensis adults have a pair of metasternal glands (MGs) that open to the ventral metathorax (Brindley 1930), but their functions, as well as the composition of their secretion, are unknown for this species. All triatomine species studied to date also possess a pair of Brindley's glands, opening dorso-laterally between the thorax and abdomen, which secrete isobutyric acid as the most abundant compound; this secretion probably plays a role in alarm and/or defense (Ward 1981; Cruz López et al. 1995; Manrique et al. 2006). Other reports for several triatomine species suggest that the compounds produced by Brindley's glands are involved in the sexual communication (Fontán et al. 2002; Rojas et al. 2002; Guerenstein and Guerin 2004). Recently, Manrique et al. (2006) and Pontes et al. (2008) suggested that the MG secretion of *T. infestans* and *R. prolixus* is involved in sexual communication. These authors identified several highly volatile ketones and alcohols that are produced by MGs of these species, and showed that the contents are emitted by adults during copulation. Moreover, Crespo and Manrique (2007) and Pontes et al. (2008) have shown that MG volatiles are relevant for mating success. However, odor-mediated orientation mechanisms that could promote encounters between adults of triatomines are not known. Therefore, we analyzed the behavior of adult *T. brasiliensis* in the presence of air currents laden with volatiles emitted by both sexes, searching for oriented responses. Additionally, we tested whether the compounds emitted by the MGs of females are necessary for the expression of orientation responses by males. Moreover, the ability of these compounds to elicit responses from the antennae of males was studied by means of gas chromatography-electroantennographic detection (GC-EAD) assays. Finally, the full identities of 14 of the most abundant volatile compounds produced by the MGs of *T. brasiliensis* females and males are reported, with special attention to those compounds with GC-EAD activity.

Methods and Materials

Insects T. brasiliensis were reared in the laboratory at 26± 2°C and 60±10% RH, and fed on live chickens. The insects were sorted by sex as fifth instar larvae (Espínola 1966), and maintained apart until the experiments were carried out. Adults were fed weekly and kept in plastic containers, which had a piece of folded filter paper as substrate. Identification of volatiles and behavioral assays were performed with 30 d old virgin adults starved for 2 wk. All insects used for this study were exposed to 12:12 hL/D cycle at least 3 d before experiments.

Olfactometer Bioassays A two-choice "T" olfactometer was used to test orientation responses of adult insects (Fig. 1). The olfactometer was made of acrylic (two 21.5 cm arms and a 33.5 cm common stem, arm section 24.0 cm^2). The apparatus was positioned horizontally in an experimental room under the same temperature conditions as described for rearing insects. All experiments were performed during the first half of scotophase. A piece of filter paper covered the base of the olfactometer in order to allow the insects to walk easily. Filtered air was drawn through the olfactometer by a fan connected to the release chamber. Air was purified by using charcoal filters located before the stimulus chamber of each arm of the olfactometer. The filters consisted of 10-ml glass pipettes filled with granular activated charcoal (4–8 mesh, Sigma, USA). The air-flow was maintained at a speed of 10 cm/s as measured by an anemometer (Testo, Germany) at the end of each arm, which was connected to a small polyacrylic chamber (6×6×3.5 cm) used for presenting the odor sources.

Filter papers were changed after each test to avoid bias due to chemical contamination left from walking insects. The stimuli associated with each olfactometer arm were alternated after three repetitions to avoid directional bias effects. The olfactometer was washed with distilled water and dried with tissue paper before each experiment.

Bioassay Calibration Tests were performed to evaluate whether the olfactometer constructed *ad hoc* presented any intrinsic bias. For this, *T. brasiliensis* adults were tested under two conditions: a) no air currents associated, and b) both arms associated with clean air currents.

Olfactometer Tests The olfactometer was used to test whether adult *T. brasiliensis* showed orientation responses when exposed to air currents associated with volatiles from adult males or females. The orientation of insects of both sexes was tested by offering individual bugs a choice between an arm associated with volatiles from two adult insects of the same gender or another that presented a clean air current. For each series, 30 individuals were tested

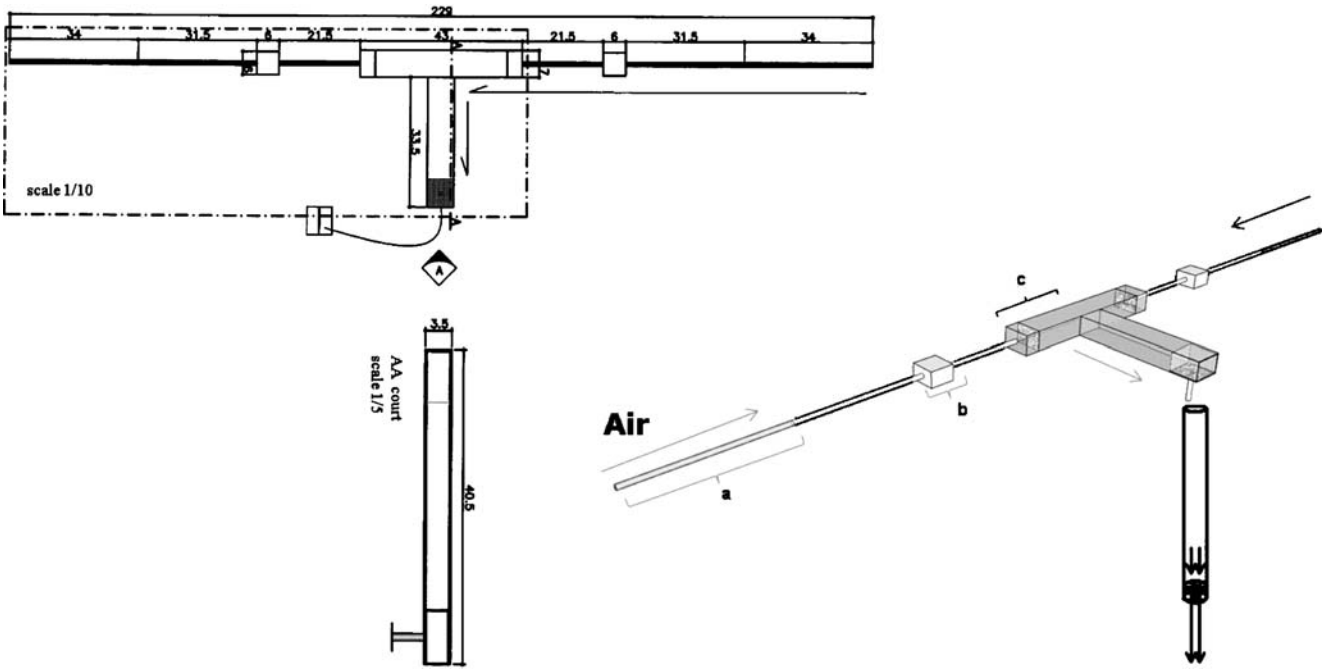

Fig. 1 Schematic drawing of the T-olfactometer used to test orientation responses in adults of *Triatoma brasiliensis*. **a** activated charcoal filter, **b** stimulus chamber, and, **c** arm. Dimensions are given in cm

against odor-laden or clean air currents. A single *T. brasiliensis* adult was introduced gently into the release chamber and kept there for acclimatization for 10 min. Subsequently, the entrance door of this compartment was opened, and the behavior of the insect was recorded for 15 min. Insects that did not express a choice after 15 min from the beginning of the assay were discounted. Test and stimulus insects were used only once.

To determine preferences, the first choice of each insect, i.e., which arm of the olfactometer that was chosen, was recorded. For this, we adopted the following criterion: an insect made a choice every time that its whole body passed the boundary between the main trunk and the corresponding arm of the olfactometer. Choices were statistically analyzed by means of a binomial test.

The following experimental series were performed to investigate whether male or female *T. brasiliensis* adults orientate to conspecific odor-laden air currents: a) female bugs exposed to male odor-laden air currents; b) female bugs exposed to female odor-laden air currents; c) male bugs exposed to female odor-laden air currents; d) male bugs exposed to male odor-laden air currents; and e) male bugs exposed to nymph odor-laden air currents.

Because of the positive response of males towards female and male odors, two complementary series of assays were conducted to elucidate further aspects of their behavior. The series tested were: f) male bugs confronted with an air current laden with volatiles from two males with the orifices of their MGs occluded with paraffin; and g) male bugs confronted with an air current laden with volatiles from two females with the orifices of their MGs occluded with paraffin. In order to test whether the response of males was due to the occlusion manipulation, two additional control assay series were performed, as follows: h) male bugs were confronted with an air current laden with volatiles from two sham-treated males to which paraffin was applied without occluding their MG orifices; and i) male bugs were confronted with an air current laden with volatiles from two sham-treated females to which paraffin was applied without occluding their MG orifices. Because of the absence of significant orientation by females in response to volatiles from adults, a positive control was performed to confirm that this apparatus was adequate for testing orientation by female bugs. In this experimental series, the females had to choose between one arm associated with mouse odors from two laboratory-reared *Mus musculus* newborns vs. a clean air current.

Identification of Compounds Produced by Metasternal Glands In order to obtain glands for the sampling of volatiles, insects were first kept at −18°C for 7 min to avoid disturbance during the dissection. They were then secured with flour-based modeling dough (Faber-Castell, Brazil), leaving their thorax and abdomen exposed. These preparations were placed into an ice-cold phosphate buffer solution to minimize volatilization of MG secretions. Immediately after mounting the insects, a microsurgical knife was used to remove the MG together with its reservoir and a piece of

cuticle surrounding the gland opening (ca. 0.25 mm^2). Samples of 10 glands were stored in 2-ml vials closed with Teflon®/silicone-lined caps and kept in an ice bath until the end of the subsequent dissections. To exclude any compounds that were not found exclusively in the MGs, we prepared control samples with pieces of tissue and cuticle from other parts of the thorax. Female and male samples were stored in a freezer at −18ºC for not more than 10 d for subsequent analysis. No change of the chemical profile was observed after storage, compared with freshly prepared samples.

Gland samples were sonicated (40 kHz, Thorton, Inpec Eletrônica, Brazil) for 5 min and then heated at 50°C for 30 min. A solid phase microextraction (SPME) fiber (2 cm, DVB/CAR/PDMS-50/30 µm, SUPELCO, USA) was exposed to the headspace of the samples for 10 min at 50°C prior to analysis by gas chromatography with mass-spectrometric detection (GC-MS Shimadzu 17A coupled to a Shimadzu 5050A). The desorption time in the injection port of the GC was 1 min. Helium was used as carrier gas (30 cm/s), and injections were splitless for 1 min. GC injector and transfer line temperatures were 230°C and 250°C, respectively. The ionization energy was 70 eV. The oven program was 40°C for 5 min, 3°C/min to 120°C, and 15°C/min to 200°C using a SupelcoWax-10 column (30 m×0.25 mm i.d.×0.25 µm film; Supelco, USA). Tentative identification of volatiles was based on the comparison of retention index (RI, Kováts 1965) and mass spectra with data from the literature and a spectral library (NIST-02). All tentative identifications were confirmed by peak enhancement in co-injections with authentic synthetic samples (Pontes et al. 2008).

The configuration of chiral compounds was determined by GC with flame-ionization detection (FID; Shimadzu 17A) analysis and GC-MS analysis (Shimadzu 17A coupled to a Shimadzu 5050A). Gland samples were heated at 50°C for 30 min. An SPME fiber (2 cm, DVB/CAR/PDMS-50/30 µm) was exposed to the headspace of the glands for a varying time interval depending on the abundance of compounds. The desorption time in the injection port of the GC was 1 min. For the GC-FID, the following settings were used: helium was used as the carrier gas (31 cm/s), and injections were splitless for 1 min. Injector and detector temperatures were 225°C. All enantioselective GC was performed isothermally using a CYCLOSILB column (30 m×0.25 mm i.d.×0.25 µm film, J & W Scientific, USA). The column temperature varied from 30°C for 2-butanol to 110°C for 1-phenylethanol. Because of the occurrence of overlapping peaks of different compounds when using the enantioselective GC-FID set-up described above, additional enantioselective GC-MS analysis was carried out according to the protocol for achiral analysis, but with a GammaDex 225 column (30 m×0.25 mm i.d.× 0.25 µm film, Supelco USA). As for the CYCLOSILB column, isothermal conditions were applied, with column temperatures varying from 30°C to 110°C. The retention times were compared with synthetic standards, and co-injections with peak enhancement were carried out to confirm the configuration of all chiral compounds.

Chemicals 2-butanone, 3-pentanone, (2*R*)-butanol, (2*S*)-butanol, 2-methyl-1-propanol, 3-pentanol, (2*S*)-pentanol, 3-hexanol, (2*S*)-methyl-1-butanol, 1-heptanol, and (1*R*)-phenylethanol were purchased from Sigma-Aldrich (Sweden and Brazil). The remaining compounds were synthesized as described below:

For all synthesized compounds ^{1}H-NMR and ^{13}C-NMR spectra of $CDCl_3$ solutions were recorded at 500 MHz and 125 MHz, using a Varian Unity spectrometer. Chemical shifts were expressed in ppm in relation to tetramethylsilane, multiplicity (s, singlet; d, doublet; t, triplet; and m, multiplet), coupling constants (Hz) and number of protons. The starting materials employed were obtained from commercial suppliers and used without further purification. Mass spectra (70 eV, EI) of synthetic compounds were obtained with an HP 6890 GC interfaced to an HP 5973 mass selective detector (Hewlett Packard, Palo Alto, CA, USA), using helium as the carrier gas. The GC was equipped with a BPX-70 column (30 m×0.25 mm, ID 0.25 µm, SGE, Australia). Enantioselective GC was performed with an HP 5890 GC (Hewlett Packard, Palo Alto, CA, USA) fitted with a CYCLOSILB column (30 m× 0.25 mm, ID 0.25 µm, J & W Scientific, USA). Chromatography on silica gel was carried out using a medium pressure liquid chromatography (Baeckström et al. 1987). All chemicals used as starting materials in the syntheses were used as delivered from Sigma-Aldrich (Sweden) and Alfa Aesar (Germany). Anhydrous solvents were used, and reactions were carried out under nitrogen when appropriate.

(2R)-Pentanol This chiral alcohol was obtained by resolution of racemic 2-pentanol with the use of *Candida antarctica* B lipase.

Racemic 2-pentanol (20 g, 0.23 mol), vinyl acetate (70 g, 0.81 mol), and *Candida antarctica* B lipase (300 mg) were added to dichloromethane (*ca*. 150 ml). After 160 min, the enzymes were removed by filtration, the solvent removed *in vacuo*, and pentane was added. The unreacted (2*S*)-pentanol was separated from the formed (2*R*)-pentyl acetate by chromatography. A fraction containing 1.66 g of the acetate was isolated. A solution of (2*R*)-pentyl acetate (1.50 g, 11.5 mmol), KOH (1.0 g, 18 mmol) and MeOH/water (20 and 3 ml, resp.) was stirred at room temperature (RT) over night. Most of the methanol was removed *in vacuo*, water was added, and the product was extracted ×3 with diethyl ether, washed with brine, dried over $MgSO_4$, and the diethyl ether was removed on an ice bath *in vacuo*

to yield 710 mg (8.1 mmol) of a slightly yellow product. Chemical and enantiomeric purities were 99%.

3-Methyl-2-hexanol This alcohol was prepared by a Grignard reaction between 2-bromopentane and acetaldehyde. Since all isomers were present in similar amounts in the glands, no stereospecific synthesis was carried out:

A round-bottomed flask was charged with magnesium (3.5 g, 0.144 mol), iodine (1 crystal), and diethyl ether (50 ml). To the mixture, 2-bromopentane (15.9 g, 0.10 mol, 13.0 ml dropwise addition via syringe) was added, maintaining spontaneous reflux to form the Grignard reagent. Thereafter, the reflux was continued for 20 min. After cooling to 0°C, acetaldehyde (6.16 g, 0.14 mol, 10.0 ml) was added to the mixture by syringe. After reflux for 20 min, the reaction was quenched by addition of ice-water followed by HCl (10%, aq.) until all precipitate was dissolved. The product was extracted ×4 with diethyl ether and washed twice with bicarbonate (sat, aq.) and brine, dried over $MgSO_4$ and concentrated *in vacuo* to yield 7.73 g of a slightly yellow oil. Some 3-methyl-2-hexanone had been formed, and to remove this impurity, the crude product was treated with sodium borohydride (0.30 g, 8 mmol) dissolved in aqueous ethanol (50%, 25 ml). The crude product (7.73 g) was added dropwise at 0°C while stirring. Stirring was continued at RT over night. Potassium carbonate was added until the mixture became transparent and saturated. The product was extracted ×3 with diethyl ether, washed twice with brine, dried over $MgSO_4$ and solvents were removed *in vacuo* to yield 5.60 g (48%) of a slightly yellow oil, which was purified by chromatography. A sample of 3.38 g product of 99% purity (diastereomeric ratio ca. 2:3) was isolated. MS: 39(5), 41(11), 42(5), 43(24), 44(9), 45(100), 55(16), 56 (4), 57(5), 59(3), 69(4), 70(29), 71(5), 83(6), 98(3), 101(4). ^{1}HNMR: δ: 3.65-3.70 (m, 1 H), 1.0-1.6 (m, 5H), 1.12-115 (d, 3H, J=6.3 Hz) 0.85-0.92 (m, 6H) (the peaks of two diastereomers overlapped to a large extent and that made the NMRs difficult to interpret.) ^{13}CNMR: δ: 72.0, 71.7, 40.0, 39.7, 35.1, 35.0, 20.6, 20.6, 20.5, 19.5, 14.7, 14.6, 14.3 ppm (one overlapping signal).

6-Methyl-1-heptanol This achiral alcohol was synthesized from 3-methyl-1-butylbromide via a Grignard reaction with oxetane (Bestmann and Vostrowsky 1974). MS and NMR data corresponded with literature data (Reiter et al. 2003; Tang et al. 1995).

(4R)-Methyl-1-hexanol The reaction of (3*S*)-citronellyl bromide in a modified Lemieux-von Rudloff periodate-permanganate oxidation (Lemieux and Rudloff 1955; Overberger and Kaye 1967; Higashimura et al. 1983; Chen et al. 2008) followed by an lithium aluminum hydride (LAH) reduction yielded the product in 99% enantiomeric purity.

(3*S*)-Citronellyl bromide (1.50 g, 6.84 mmol) was dissolved in 50% acetone in water (50 ml). $NaIO_4$ (5.0 g, 20.7 mmol) and $KMnO_4$ (0.2 g, cat) were dissolved in water (50 ml) and added slowly to the solution of (3*S*)-citronellyl bromide at 0°C. The mixture was stirred at RT overnight (16 h). After confirming full conversion (GCMS), the product mixture was filtered *in vacuo*. Most of the acetone was removed *in vacuo*, and the remaining pink solution was acidified to pH≈2 by HCl (10%, aq.). The acidic solution was stirred at RT for 30 min. The orange solution was extracted ×3 with diethyl ether, washed twice with brine, dried over $MgSO_4$, and concentrated *in vacuo* to give a copper-colored oil: 1.45 g (101%). The crude (4*S*)-6-bromo-4-methylhexanoic acid (1.45 g) was dissolved in diethyl ether (40 ml) and was added dropwise to a solution of LAH (1.9 g, 50 mmol) in diethyl ether (50 ml) at steady reflux. The mixture was refluxed for 1 h, and then quenched by addition of Baeckström's reagent (sodium sulfate deca-hydrate/celite, Baeckström et al. 1991). When the suspension had changed from grey to white, the solids were removed by filtration *in vacuo*. The solvents were removed *in vacuo* to yield a colorless oil of 63% purity (612 mg, 49%). After purification by chromatography a fraction of 99% purity (257 mg, 99% enantiomeric purity) was obtained. Spectroscopic data correlated well with literature data (NIST 2002; Larsson et al. 2001).

(4R)-Methyl-1-heptanol This chiral alcohol was synthesized from (2*R*)-methyl-1-pentanol by malonate ester synthesis (Barth and Effenberger 1993; Hedenström et al. 2002; Tai et al. 2002).

Racemic 2-methyl-1-pentanol (16.0 g, 0.16 mol) and vinyl acetate (56.0 g, 0.65 mol) were dissolved in dichloromethane (250 ml) at RT. Amano PS-D lipase (800 mg) was added while stirring. The reaction was monitored hourly by enantioselective GC. When the (2*R*)-methyl-1-pentanol remaining in the mixture had an *e.e.* of 96% (at ca. 75% conversion), the lipase was filtered off. The mixture was concentrated *in vacuo*, and the alcohol (4.0 g, 50%) was isolated by chromatography.

(2*R*)-Methyl-1-pentanol (3.40 g, 33.3 mmol) was dissolved in a mixture of p-toluene sulfonyl chloride (6.8 g, 35.7 mmol) in dry pyridine (20 ml). The mixture was stirred at RT for 6 h and then quenched by pouring into ice-water (40 ml). The product was extracted ×3 with diethyl ether, the organic phase was washed with copper sulfate solution (10%, aq.), bicarbonate (sat., aq.), and brine, dried over $MgSO_4$, and concentrated *in vacuo* to yield a pale yellow oil of 79% purity (6.74 g). MS: 39(6), 41(11), 42(5), 43(19), 55(11), 56(28), 63(4), 65(23), 69(28), 71(4), 77(4), 83(4), 84(100), 85(8), 89(8), 90(5), 91(93), 92(27), 93(4), 107(8), 108(5), 139(5), 155(100), 156(18), 157(21), 172 (13), 173(43), 174(4).

The crude (2*R*)-methyl-1-pentyl tosylate (6.6 g, 25.8 mmol) was added to a solution of lithium bromide (12.7 g, 146 mmol) in acetone (100 ml). The mixture was refluxed overnight and then concentrated. Water was added, and the mixture was extracted ×4 with pentane, the organic phases washed twice with water and dried over $MgSO_4$. The solvent was removed *in vacuo* to yield a pale yellow oil of 57% purity (6.6 g, 89%). After chromatography, a fraction of 2.89 g was used as such in the next step. MS: 39(13), 41(35), 42(12), 43(7), 55(16), 56(10), 57(9), 69(12), 71(21), 84(8), 85(100), 86(10), 121(7).

Diethyl malonate (2.84 g, 17.7 mmol) was added to a solution of sodium ethoxide (1.15 g in 10 ml ethanol), and the mixture was refluxed for 15 min. 2-Methylpentyl bromide (2.80 g, 17.1 mmol) was added dropwise at RT while stirring, whereafter the mixture was refluxed for 1 h. The solution was acidified at RT by acetic acid (3 drops), and then diluted by water (10 ml) and diethyl ether (10 ml). The organic phase was separated, and the water phase was extracted ×4 with diethyl ether. The organic phases were washed twice with brine, dried over $MgSO_4$, and the diethyl ether was removed *in vacuo*. The copper-colored residue (1.76 g) was added to a solution of potassium hydroxide in ethanol (30 ml, 0.14 g/ml), and the mixture was refluxed for 4 h. After removal of most of the ethanol *in vacuo*, water was added, and the solution was washed twice with diethyl ether, acidified to pH≈1 (HCl, conc.), extracted ×3 with diethyl ether, washed with water and brine, concentrated *in vacuo*, and heated at 180° C for 2 h. A solution of sodium carbonate (sat., aq.) was added at RT, and the aqueous solution was washed with diethyl ether, acidified (HCl, conc.), extracted with diethyl ether, washed with water and brine, dried over $MgSO_4$, and concentrated *in vacuo* to yield a pale brown oil (203 mg). The carboxylic acid intermediate (203 mg, 1.4 mmol) was added in diethyl ether (5 ml) by syringe to a solution of LAH (65 mg, 1.7 mmol) in diethyl ether (5 ml). The mixture was stirred for 1 h, whereafter Baeckström's reagent was added. When the reaction mixture turned white, the precipitate was removed by filtration *in vacuo* and the solvent removed *in vacuo*. The yield of a colorless oil of 99% purity was 141 mg (6%, 98% enantiomeric purity). Spectroscopic data correlated well with literature data (NIST 2002; Tai et al. 2002). MS: 39(10), 40(2), 41(46), 42(17), 43(44), 44(3), 45(3), 53(4), 54(1), 55(44), 56(56), 57(12), 60(1), 67(5), 68(5), 69 (100), 70(54), 71(16), 82(3), 83(13), 84(81), 85(6), 87(3), 97(5), 112(1). ^{1}HNMR: δ: 3.63 (m, 2H), 1.1-1.6 (m, 9H), 0.87 (m, 6H), ^{13}CNMR: δ: 63.7, 39.5, 33.1, 32.6, 30.6, 20.3, 19.8, 14.6 ppm. Total yield from (2*R*)-methyl-1-pentanol was 4%.

Gas Chromatography Coupled to Electroantennographic Detection

Headspace collections were made over 8 excised MGs of females by means of SPME, using the same fiber type as described for the chemical analyses. The volatiles collected were analyzed by GC-EAD using the same GC-setup as for GC-FID analyses, fitted with a SupelcoWax-10 column (30 m×0.25 mm ID; 0.25 μm film), and coupled to an electroantennographic setup (Syntech, Hilversum, The Netherlands). Recordings of responses to volatiles eluting from the GC column were obtained from the antennae of 8 different males in separate experiments. An antenna was isolated from the head by cutting at the antennal base. The proximal end of the isolated antenna was inserted into a borosilicate glass micropipette containing 0.1 M KCl solution, serving as the reference electrode. The distal end of the antenna was inserted into another glass micropipette filled with 0.1 M KCl solution after the tip of the segment was cut off. Ag-AgCl wire was used to maintain electrical continuity between the electrodes, and a high input impedance headstage preamplifier (Syntech, Kirchzarten, Germany). The EAG signals through the preamplifier were further amplified and processed with a PC-based signal processing system (Syntech, Kirchzarten, Germany). The digitized signals from the FID and antenna were analyzed by means of the EAD2000 software (ver. 2.3, Syntech, Hilversum, The Netherlands).

Results

Olfactometer Bioassays In the negative control experiments, neither male nor female insects showed significant orientation to either arm of the olfactometer when no air current or two clean air currents were presented. Female insects did not show a significant preference for male or female odor-laden air currents (Fig. 2) but were significantly attracted to the odor emitted by mice (Fig. 2, $P<0.001$) in the positive control experiments, showing that the apparatus is capable of evincing orientation responses by these bugs. *T. brasiliensis* males, in contrast to females, significantly preferred the arm of the olfactometer associated with male or female odors (Fig. 3, $P<0.01$ and $P<0.001$, respectively). When the MGs of the female insects used as stimuli were occluded, males did not show a significant orientation (Fig. 4). However, sham-treated females promoted the orientation of males in the olfactometer (Fig. 4, $P<0.01$). The occlusion of the MGs of males used as sources of stimuli did not promote a change in the orientation of males in the olfactometer (Fig. 4, $P<0.01$).

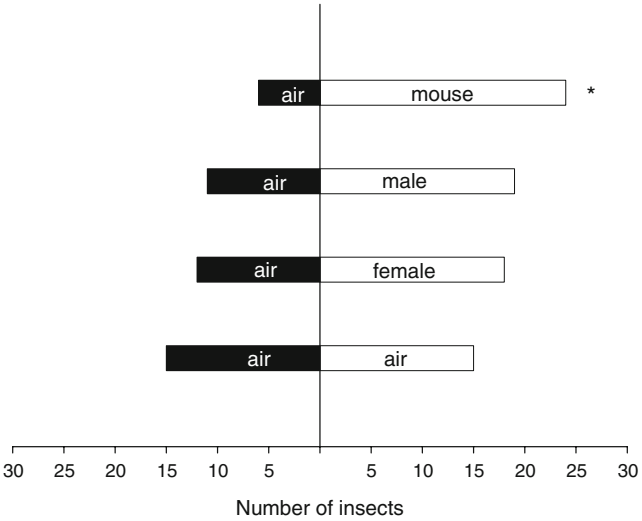

Fig. 2 Response of *Triatoma brasiliensis* females to different stimuli in an olfactometer. Significant differences between treatments were determined by a binomial test (*$P<0.001$)

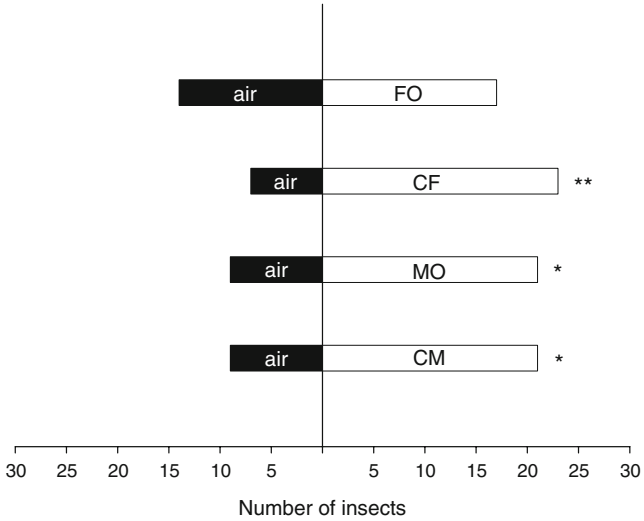

Fig. 4 Responses of *Triatoma brasiliensis* males to different stimuli in an olfactometer. MO=males with occluded metasternal glands (MGs), CM=control males treated with paraffin on a different part of their body surface, FO=females with occluded MGs and, CF=control females treated with paraffin on a different part of their body surface. Significant differences between treatments were determined by a binomial test (*$P<0.01$ and **$P<0.001$)

Identification of Compounds Produced by MGs The MGs of *T. brasiliensis* are the sources of a complex mixture of volatiles. In total, sixteen compounds were identified, with the most abundant compounds being 3-pentanone, 3-pentanol, and (4*R*)-methyl-1-heptanol (Table 1). The configurations of the chiral compounds varied; 2-methyl-1-butanol was present as the *S*-enantiomer, 4-methyl-1-hexanol, 4-methyl-1-heptanol, and 1-phenylethanol were present as the *R*-enantiomers, while 3-hexanol and 3-methyl-2-hexanol were present in all isomeric forms. For 2-butanol and 2-pentanol, problems with co-elution and/or low abundance of the compounds prevented us from confirming the configuration.

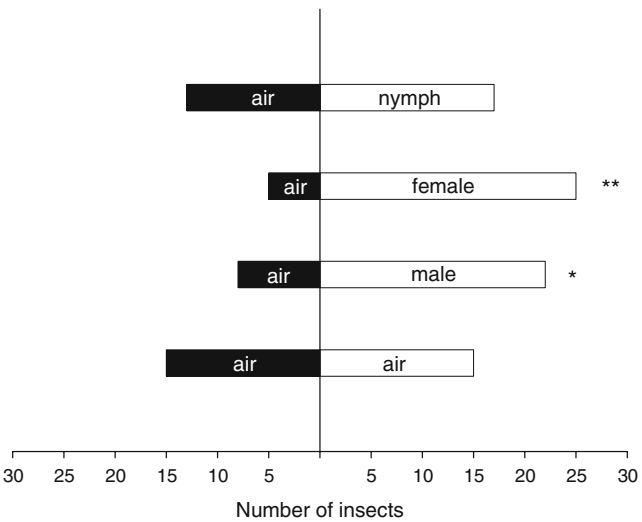

Fig. 3 Response of *Triatoma brasiliensis* males to different stimuli in an olfactometer. Significant differences between treatments were determined by a binomial test (*$P<0.01$ and **$P<0.001$)

Gas Chromatography Coupled to Electroantennographic Detection Our GC-EAD recordings showed that the antennae of males of *T. brasiliensis* respond to a series of volatile compounds produced by the MGs of females (Fig. 5). Responses to 3-pentanone were consistently observed in all recordings and were normally the strongest. In addition, 75% of the recordings presented strong responses to 4-methyl-1-heptanol, and responses also were observed for 2-methyl-1-butanol and 1-phenylethanol. It is worth highlighting that one of the compounds reported here as unknown (RI=1,156) was capable of promoting responses from 3 out of 8 antennae tested (Fig. 5).

Discussion

Orientation in response to odor-laden air currents is a widespread mechanism that mediates sexual encounters between male and female insects. However, no cases of anemotactic orientation to sexually related signals are known to date for triatomine bugs. Our report presents significant evidence showing that *T. brasiliensis* males express oriented responses to air currents associated with volatiles from conspecific adults. These anemotactic responses are apparently stronger when males are con-

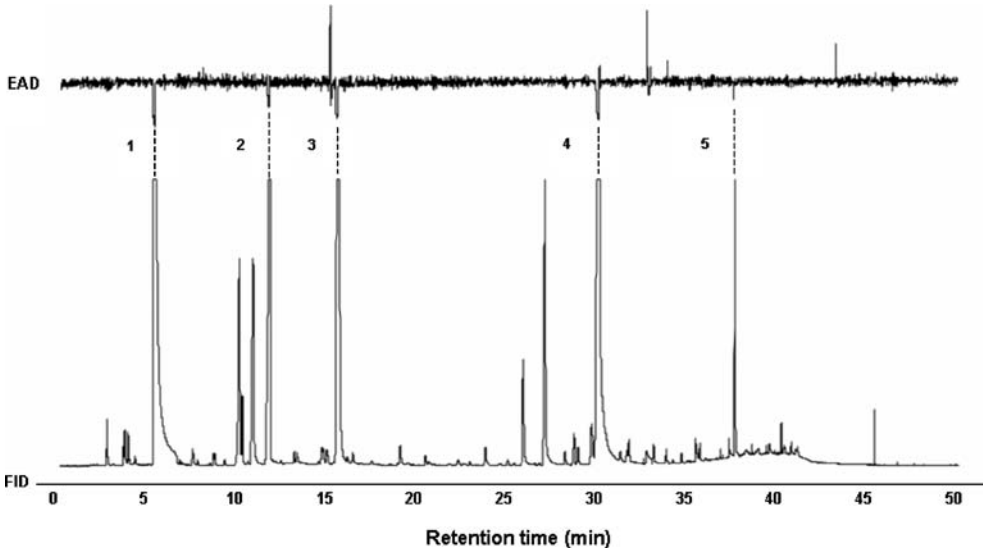

Fig. 5 Gas chromatogram (FID) and corresponding electroantennogram (EAD) traces from one antenna of a *Triatoma brasiliensis* male, in response to a headspace collection of metasternal gland (MG) volatiles. Active compounds are represented with numbers: 1) 3-pentanone, 2) unknown, 3) 2-methyl-1-butanol, 4) 4-methyl-1-heptanol, and 5) 1-phenylethanol

fronted by air currents laden with female volatiles rather than with male odors. This finding suggests that males are attracted by odors emitted by distant females, and exploit air currents to define the direction of their responses. This may represent a putative mechanism to mediate encounters between adults of different gender, and suggests that a sexual pheromone is produced by females.

Our results showed that MGs of females emit a volatile signal that is necessary to mediate the orientation expressed by males. On the other hand, the preference observed when male insects were confronted with male odor was not affected by the occlusion of MGs. Therefore, male to male orientation is apparently not mediated by signals produced by these glands. As females did not show oriented responses to air currents laden with odors from adult bugs, we propose that *T. brasiliensis* males are the only gender attracted to sexual signals.

Table 1 Compounds identified in metasternal glands of *Triatoma brasiliensis*

RI[a]	Rel. amount[b]	Compound[c]
909	*/**	2-butanone
987	*****	3-pentanone
1,033	*	(2*S*)-butanol[#]
1,113	*	2-methyl-1-propanol
1,127	****/*****	3-pentanol
1,136	*/**	(2*R*)-pentanol[#]
1,156	***	unknown
1,205	*	(3*R*)-hexanol and (3*S*)-hexanol
1,215	**/***	(2*S*)-methyl-1-butanol
1,287	*	3-methyl-2-hexanol[##]
1,421	*	(4*R*)-methyl-1-hexanol
1,446	*/**	1-heptanol
1,500	*	6-methyl-1-heptanol
1,508	****/*****	(4*R*)-methyl-1-heptanol
1,548	*	unknown
1,816	*/**	(1*R*)-phenylethanol

[a] Retention indices calculated according to Kováts (1965). [b] Relative amounts: *****= >20%, ****=10-20%, ***=5-10%, **=2-5%, *= >2% of total compounds (GC peak areas). [c] Compound identifications: [#] =tentative configuration, not conclusive results from peak enhancement experiments, [##] =all isomers present in glands

The use of antennae of male insects as detectors for electrophysiologically active compounds made it possible to demonstrate that several substances produced by MGs stimulate male receptors. Specifically, 3-pentanone and (4*R*)-methyl-1-heptanol, elicited strong responses in most preparations, while (2*S*)-methyl-1-butanol, (1*R*)-phenylethanol and two unknown compounds (RI=1,156 and RI=1,548, Table 1) elicited weaker responses from antennae of males. Whether or not other components of the female MG secretion are capable of stimulating antennal receptors needs to be determined. It is worth mentioning that while the secretions produced in the MGs of females and males were similar according to our GC-MS analyses, the responses observed when males were tested in olfactometers suggest that the products of male and female MGs are different. This is supported by the two occlusion experiments, as the occlusion of male MGs did not affect the orientation of males to the air currents laden with volatiles from other males.

It has been proposed that the secretion of the MGs of triatomines is involved in mediating sexual communication (Manrique et al. 2006; Pontes et al. 2008). The identification of the volatile compounds detected over the headspace of *T. brasiliensis* MGs corroborated that these glands produce ketones and alcohols in triatomines. The fact that 3-pentanone is the most abundant compound produced by MGs is coincident with the results presented by Manrique et al. (2006) for *T. infestans*. The relatively less volatile 4-methyl-1-heptanol, that appears to be characteristic of the

latter species, is reported here for the first time. Our GC-EAD results suggest that both substances could be involved in mediating behavioral responses of the kind described in this study. Several other compounds reported here (2-butanone, 2-pentanone, (2*S*)-butanol, 3-methyl-2-butanol, 3-pentanol, (2*S*)-pentanol, and (3*S*)-hexanol) were previously detected in the MGs of other blood-sucking bugs (Manrique et al. 2006; Pontes et al. 2008). All compounds found in the MGs except 4-methyl-1-hexanol, 6-methyl-1-heptanol, and 4-methyl-1-heptanol have been reported previously in other insect species (El-Sayed 2008). It is relevant to highlight that capturing volatile compounds by means of SPME allowed us to perform successful GC-EAD recordings, confirming that this odor extraction technique is adequate for the preparation of odor samples for electrophysiological studies.

Baldwin et al. (1971), as well as Manrique and Lazzari (1995), have shown that *R. prolixus* and *T. infestans* males aggregate around mating pairs, and suggested that this behavior is mediated by chemical signals. In addition, Vitta and Lorenzo (2009) have shown recently that *T. brasiliensis* males react to the presence of competitors by showing increased mate-guarding responses. Furthermore, these authors described that mating duration can be shortened if males copulate with previously mated females. Whether these changes in male behavior are triggered by chemical signals is not known. Crespo and Manrique (2007) and Pontes et al. (2008) have demonstrated that the occlusion of the MGs of *T. infestans* and *R. prolixus* affects mating success in a dramatic manner. In addition, it has been suggested that the secretion from Brindley's glands is involved in sexual communication of triatomines (Fontán et al. 2002; Rojas et al. 2002; Guerenstein and Guerin 2004). Nevertheless, no behavioral evidence has been reported associating compounds from Brindley's glands with behavioral responses specifically related to sex.

Baits and traps for triatomine bugs are necessary for the early detection of reinvasion of houses after insecticide treatments, in order to avoid contact between triatomines and humans. Whether the compounds and behavioral responses described here can be exploited for the development of control tools will depend on further work testing synthetic compounds in dose-response electrophysiological and behavioral bioassays.

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changes as host colonization proceeds (Birgersson and Bergström 1989). Both males and females may contribute unique components to the pheromone blend, whereas only one sex initiates colonization (Borden 1982); hence pheromone components from the pioneer sex are emitted prior to those of the joining sex, but as multiple families accumulate at an aggregation, signaling by the pioneering and joining sexes overlaps. Additionally, either sex may produce a succession of different compounds during host colonization (Birgersson et al. 1984; Byers et al. 1984). Attacks on individual trees begin frequently at a specific height on the bole and then spread to adjacent portions over the course of a few days, and adjacent trees within infestations are typically attacked successively rather than simultaneously (Gara and Coster 1968; Berryman 1982; Schlyter et al. 1987a). Hence, at any one time, the composition and release rate of the emitted pheromone blend will vary spatially, both along the length of the infested bole as well as among adjacent, attacked trees. Pheromones arising from adjacent rather than identical points in space may send a distinctly different message to host-seeking beetles, and thus they might differently influence the location where a beetle ultimately lands. However, few studies of bark beetles have examined the effect of separating the release points of aggregation pheromone components (Byers 1987).

The southern pine beetle, *Dendroctonus frontalis* Zimmermann (Coleoptera: Scolytidae), is an aggressive bark beetle that is native to the southeastern United States, Arizona, Mexico, and parts of Central America (Payne 1980). Infestations typically occur across the landscape in spatially discrete, rapidly expanding "spots" in which adjacent trees are attacked in succession by beetles arriving from the surrounding forest as well as by re-emerging parent and brood adults from within the infestation (Gara 1967; Gara and Coster 1968). Aggregation is mediated by a complex of semiochemicals: initially-attacking females release the attractive pheromone component frontalin, which is enhanced synergistically both by monoterpene kairomones (particularly α-pinene) released by the host pine and (+)-*endo*-brevicomin from secondarily-arriving males (Smith et al. 1993; Sullivan et al. 2007). Additionally, the female-produced pheromone component *trans*-verbenol may substitute for host monoterpenes when the latter are at low levels, and may play an important role in initiating host colonization (Renwick and Vité 1969; Payne et al. 1978a). *Dendroctonus frontalis* also produces several compounds (such as verbenone and myrtenol) that inhibit response to attractants, and one or more of these may function as antiaggregation pheromones for this species (Smith et al. 1993; Sullivan 2005).

There are divergent data sets on the behavioral activity of *endo*-brevicomin with *D. frontalis*. Studies performed with racemic *endo*-brevicomin indicated that this compound acts as an attractant antagonist (Vité and Renwick 1971; Payne et al. 1978a; Salom et al. 1992). In contrast, baits composed of pure (+)-*endo*-brevicomin (only enantiomer produced by the species) strongly enhanced attraction to combinations of frontalin and host odors (Vité et al. 1985; Sullivan et al. 2007), and led these authors to conclude that this compound was a component of the *D. frontalis* aggregation pheromone. (+)-*endo*-Brevicomin alone does not attract *D. frontalis* into traps (Sullivan et al. 2007).

Observations during trapping trials with *D. frontalis* suggested that trap spacing alone might determine whether the addition of (+)-*endo*-brevicomin increased, reduced, or did not alter catch in attractant-baited traps relative to attractant-only control traps (authors' unpublished data). We hypothesized that interacting pheromone components of bark beetles (i.e., synergists, antagonists) might convey unique information and elicit different behaviors when their sources are separated by a short distance (i.e., a few meters) rather than being either collocated or widely separated. We therefore performed tests to determine if point sources of *endo*-brevicomin could influence catch in distant traps and whether varying the distance between such point sources and attractant-baited traps could reverse the apparent activity of this pheromone component. We also performed initial studies to elucidate interactions of *endo*-brevicomin release rate with trap spacing and contrast the spatial dynamics of *endo*-brevicomin to other aggregation-mediating semiochemicals for *D. frontalis*.

Methods and Materials

Location, General Design, and Materials Trapping experiments were performed by using 12-unit funnel traps (Chemtica International, San Jose, Costa Rica) placed in mature, mixed pine/hardwood stands of the Homochitto National Forest in southwestern Mississippi (31.43° N, 91.19° W). The pine component was dominated by loblolly pine, *Pinus taeda* L., interspersed with shortleaf pine, *Pinus echinata* Mill. Adjacent pine forests were experiencing moderate levels of *D. frontalis* infestation, but according to aerial surveys no multiple-tree beetle infestations were within 1 km of the trap locations. Traps were positioned >10 m from the nearest pine and suspended (with the bottom of the trap cup 1–1.5 m above the ground) from vertical standards consisting of 1.7 cm dia. pieces of electrical conduit staked into the ground. Trap cups contained several centimeters of aqueous propylene glycol to preserve captured insects, and catch was collected at intervals of 6–18 d (longer intervals were used during periods when flying beetles were less abundant). Frontalin baits consisted of a pair of capped 400 µl-capacity LDPE

microcentrifuge tubes each loaded with 200–300 µl racemic frontalin [>95% chemical purity (contaminants with no known behavioral activity), Chemtica International]. Turpentine baits consisted of a single 250 ml-capacity brown glass bottle with a piece of 1 cm dia. cotton dental wick immersed in the liquid (200–250 ml, steam distilled from *P. taeda*; Hercules Inc., Brunswick, GA, USA) and protruding 2.5 cm through the cap. *endo*-Brevicomin was released either from glass capillaries with one heat-sealed end or from open, 100 µl-capacity glass autosampler vial inserts; these were secured open-end-up inside of an uncapped, inverted 4 or 8 ml-capacity glass screw-cap vial (Table 1). The capillaries and autosampler vial inserts were secured in the vial with a silicone GC septum that was pressed sideways into the vial mouth; the sealed end of each capillary (or the tapered tip of each insert) was inserted into a pinhole in the interior-facing edge of the septum. (+)-*endo*-Brevicomin was synthesized as described elsewhere (Sullivan et al. 2007) and was >99% enantiomerically and 95% chemically pure by GC (contaminants with no known behavioral activity). Racemic *endo*-brevicomin [PheroTech (now ConTech Inc.), Delta, BC, Canada] was 95% chemically pure (<1% *exo*-brevicomin contamination). Bait release rates were measured in a fume hood at 23±2°C, either gravimetrically for turpentine (7 g/d) and frontalin (5 mg/d) or by volume loss for *endo*-brevicomin (Table 1). Frontalin and *endo*-brevicomin baits were attached at the fourth funnel from the bottom of the trap, whereas the turpentine bottle was placed within the funnel immediately below the trap top to protect the wick from rain. During experiments, pines adjacent to traps were checked regularly for the presence of pitch tubes and other evidence of *D. frontalis* attack. If attacked pines were apparently not under mass attack (Payne 1980), traps were moved an additional 10-20 m from these trees thereby stopping further attacks; if a mass attack was observed (as occurred twice), traps were moved at least 100 m away. In both cases, data from the moved trap was used in the analyses.

Experiment 1: Displacement of (+)-endo-Brevicomin Bait from an Attractant-Baited Trap Single funnel traps were spaced >130 m apart, baited uniformly with frontalin and turpentine, and assigned one of six experimental treatments (Fig. 1a): A single (+)-*endo*-brevicomin bait was either attached directly to the trap (i.e., at 0 m), or positioned 4, 8, 16, or 32 m away, or no *endo*-brevicomin bait was assigned to the trap (in this case the closest source of *endo*-brevicomin was associated with adjacent traps and thus was >100 m away). Three lines of six traps each were established, and the six treatments were assigned initially at random to the six traps of each line and then re-randomized without replacement to any previous position for each of six successive trap collections. Thus, the experimental design was three complete Latin squares with each square consisting of six traps (columns) and six successive trapping intervals (rows). The displaced *endo*-brevicomin release devices were suspended 1.5 m above the ground on plastic garden stakes and the direction that each release device was located relative to its treatment trap (0°, 60°, 120°, 180°, 240°, or 300° from north) was assigned at random and without duplication among the six traps of each line. This directional assignment remained the same for each trap position for the duration of the experiment. The experiment was conducted 8 February–12 April 2006. Trap catches of *D. frontalis* were cube root transformed and analyzed with a mixed-model ANOVA in which treatment, date, and treatment by date effects were considered fixed, whereas square, trap within square, and treatment by square effects were regarded as random (SAS 9.0, SAS Institute Inc., Cary, NC). The cube-root transformation was used for analysis of this and all following ANOVAs of total *D. frontalis* catches because it was generally better at normalizing residuals than the weaker square root or the stronger $\log_{10}$ transformation (based on examination of residual plots), and in all comparisons the residuals from the transformed data sets did not fail the Kolmogorov-Smirnov test for a normal distribution (α=0.05). Treatment means

Table 1 Construction and elution rate of *endo*-brevicomin release devices[a]

Experiment number (release rate)	Glass insert/capillary characteristics				Elution rate[c] (mg/d ± s.d.)
	Diameter (i.d.)	Length	Height filled	Number per device	
1, 2[b], 4	1.2 mm	20 mm	10 mm	1	0.23±0.01
3 (low rate)	0.6 mm	32 mm	10 mm	1	0.045±0.007
3 (medium rate)	1.2 mm	32 mm	10 mm	3	0.52±0.03
3 (high rate)	3.7 mm	30 mm	8 mm	3	2.8±0.6

[a] Each device consisted of 1–3 glass autosampler vial inserts or glass capillaries secured open-end-up inside of an inverted, uncapped 4 ml (8 ml for the high release rate device) capacity screw-capped vial.

[b] In experiment 2, a single device was used for (+)-*endo*-brevicomin, but two devices were used for racemic *endo*-brevicomin.

[c] Measured in a fume hood at 23±2°C (N=3).

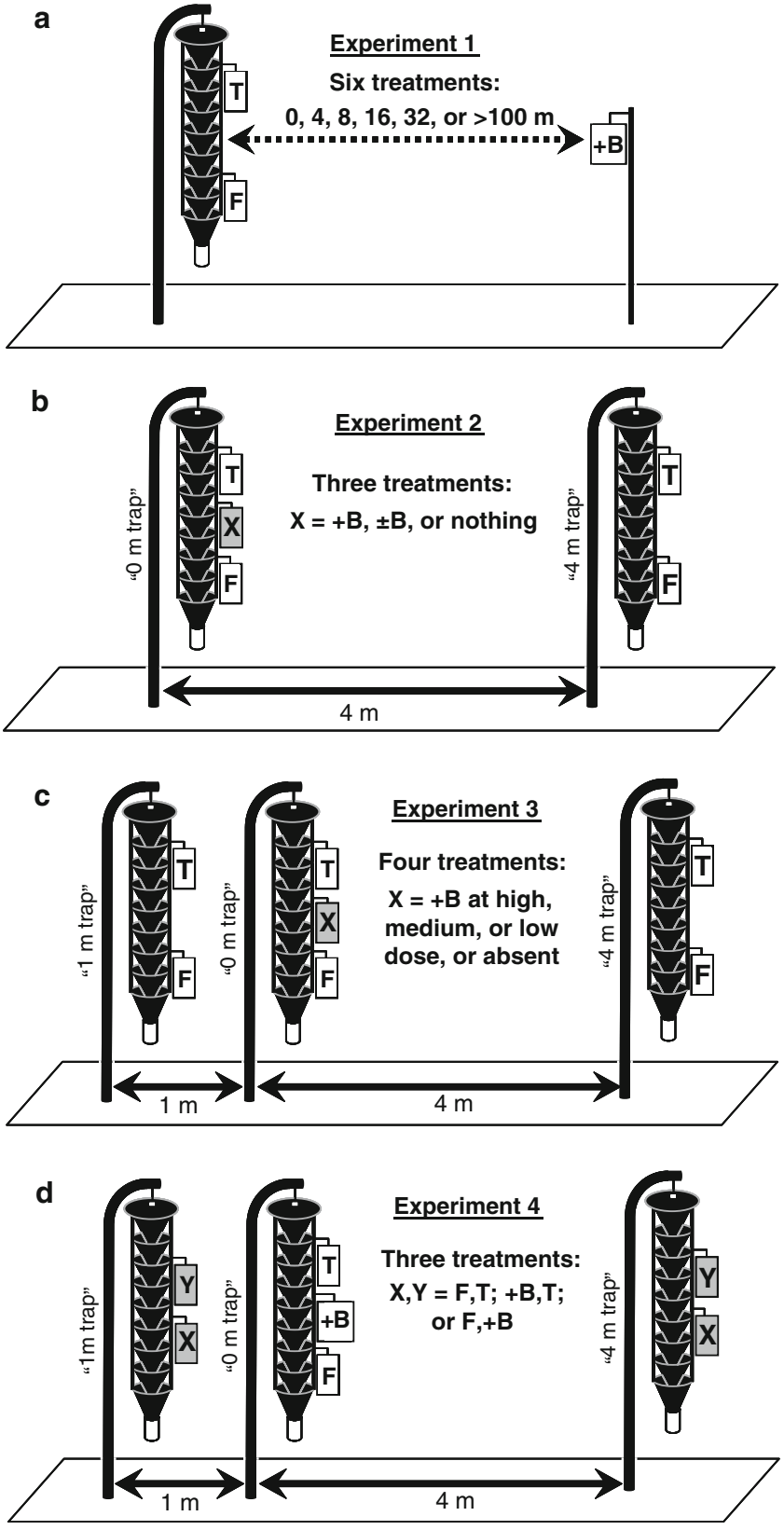

Fig. 1 Trap and bait arrangements for experiments 1–4. Rectangles represent release devices of either frontalin (F), turpentine (T), (+)-*endo*-brevicomin (+B), racemic *endo*-brevicomin (±B), or an experimentally variable bait (shaded X and Y). For the 4, 8, 16, and 32 m-distances in experiment 1, the "+B" release device was attached to the top of a plastic garden stake; for the >100 m treatment, no+B device was assigned to the trap and the closest such device was associated with an adjacent treatment. Otherwise, release devices were attached directly to traps

were compared by using Tukey's test for all pairwise comparisons (α=0.05) with treatment by square as the error term. In this and the other trapping experiments, catches for some traps and intervals were very low or zero, hence use of the above ANOVA for analysis of treatment effects on sex ratio was not possible due to the excessive numbers of missing values and/or the highly non-normal distributions of residuals. Therefore, the proportion of female *D. frontalis* trapped in each treatment was pooled within a square and subjected to a two-way ANOVA with square and treatment as factors. Proportions of females trapped were not transformed for this or other experiments since the pooled data never failed the Kolmogorov-Smirnov test (α=0.05).

Experiment 2: Pairs of Adjacent Traps Differing in endo-Brevicomin Pairs of funnel traps spaced 4 m apart and baited identically with frontalin and turpentine were established at locations separated by >100 m (Fig. 1b). One trap of each pair (the "0 m trap;" chosen by a coin toss), received either a single (+)-*endo*-brevicomin bait, two racemic *endo*-brevicomin baits, or no additional bait (three experimental treatments). The opposite trap (the "4 m trap") received no additional bait. Four lines, each with three trap pair locations, were established, and the treatments were assigned initially at random to the three pairs of each line and then re-randomized without replacement to any previous pair for each of three successive trap collections. Thus, the experimental design was four complete Latin squares with each square consisting of three adjacent trap pairs (columns) and three successive trapping intervals (rows). The experiment was run 13 December 2005–17 January 2006. The summed *D. frontalis* catch per trap pair ($\Sigma_x = X_{4m} + X_{0m}$, transformed $\sqrt[3]{\Sigma_x}$) and the difference in catch ($D_x = X_{4m}-X_{0m}$, transformed $\sqrt[3]{D_x}$), were analyzed with the mixed-model ANOVA of experiment 1. Treatment effects on the proportion of females trapped per pair and the difference in proportion of females between members of each pair were analyzed with a two-way ANOVA (as in experiment 1). Additionally, beetle responses to the two traps of each pair (X_{4m} vs. X_{0m}) were compared within each treatment by a paired *t*-test (α=0.05).

Experiment 3: Trios of Adjacent Traps with Varying endo-Brevicomin Release Rate At locations separated by >100 m, groups of three traps were erected in a straight line with the outside traps spaced 1 m and 4 m away from the middle trap (Fig. 1c). The outside traps were erected first, and then the location of the middle trap respective to either outside trap (i.e., the assignment of 4 m and 1 m-distant traps) was chosen by a coin toss. All three traps were baited with frontalin and turpentine, and the middle trap (the "0 m trap") either received no additional bait or was baited additionally with a device releasing either a low, medium, or high rate (Table 1) of (+)-*endo*-brevicomin (four experimental treatments). Two lines, each with four trap trio locations, were established, and the treatments were assigned initially at random to the four trios of each line and then re-randomized without replacement to any previous trio for each of four successive trap collections. After these initial four collections (after every treatment had been at every trio location once), the middle trap was moved 3 m in order to reverse the designation of the 1- and 4 m-distant traps within each trio. Then treatment assignments were re-randomized among trios within lines, and a second set of four successive collections was performed similar to the first. Thus, the experimental design was four complete Latin squares with each square consisting of four adjacent trap trios (columns) and four successive trapping intervals (rows). The experiment was run 16 August–23 October 2005. Total catch of *D. frontalis* per trap trio ($\Sigma_x = X_{4m} + X_{1m} + X_{0m}$; transformed $\sqrt[3]{\Sigma_x}$) as well as the differences in catch between the outside and middle traps ($D_1 = X_{4m}-X_{0m}$, $D_2 = X_{1m}-X_{0m}$; transformed $\sqrt[3]{D_x}$) were analyzed with a mixed model ANOVA in which treatment and date within square were considered fixed, and square, trio within square, and treatment by square were regarded as random effects (SAS 9.0). Date was included in the model as a fixed effect nested within square to account for replication of squares in time. Tukey's all-pairwise comparisons (α=0.05) utilized treatment by square as the error term. Treatment effects on the proportion of females trapped per trio, and the differences in proportion of females between the outer and middle traps, were analyzed with a two-way ANOVA (as in experiment 1).

Experiment 4: Trios of Adjacent Traps with Variable Baits Trap trios were established identically as in experiment 3, however, the 0 m trap was baited consistently with frontalin, turpentine, and (+)-*endo*-brevicomin, whereas the 4- and 1 m-distant traps were both baited identically with just two of these bait components and thus lacked one of (+)-*endo*-brevicomin, frontalin, or turpentine (i.e., three treatments, Fig. 1d). Three lines, each with three trap trio locations were established, and treatments were assigned initially at random to the three trios of each line and then re-randomized without replacement to any previous position for each of three successive trap collections. Thus, the experimental design was three complete Latin squares, with each square consisting of three adjacent trap trios (columns) and three successive trapping intervals (rows). The experiment was run 17 October–14 November 2005. Differences in catch between the outside and middle traps ($D_1 = X_{4m}-X_{0m}$, $D_2 = X_{1m}-X_{0m}$; transformed $\sqrt[3]{D_x}$) were compared among treatments by using the mixed-model ANOVA of experiment 1. Differences in proportion of females trapped between the outer and middle traps were subjected to a two-way ANOVA (as in experiment 1).

A corollary data set was collected to determine whether catch in the outside and middle traps differed when all three traps in each trio were baited identically. Trios as described above were established at 14 different sites, all three baits were placed at all three traps, and catch during a single 7–18 d interval was collected from each on 7 November 2005–17 January 2006. For all bait configurations, the transformed differences in catch between the outside and middle traps ($^3\sqrt{D_1}$, $^3\sqrt{D_2}$) were each tested against the null hypothesis of equality to zero with a one-sample *t*-test (two-tailed, α=0.05).

Results

Experiment 1: Displacement of (+)-endo-Brevicomin Bait from an Attractant-Baited Trap Catch of *D. frontalis* in traps baited with frontalin and turpentine was significantly influenced by trap distance from a (+)-*endo*-brevicomin release device (F=85.8; df=5, 10; P<0.001). Traps caught significantly more beetles when the (+)-*endo*-brevicomin releaser was 0–32 m distant rather than >100 m away (Fig. 2). Furthermore, catch was significantly greater when the (+)-*endo*-brevicomin release device was 4, 8, or 16 m away from the trap rather than attached directly to it. Distance of the *endo*-brevicomin releaser from the trap did not significantly alter the proportion (0.40±0.01, mean ± s.d.) of females responding (F=0.37; df=5, 10; P=0.86).

Experiment 2: Pairs of Adjacent Traps Differing in endo-Brevicomin Pairs of traps spaced 4 m apart and baited identically with frontalin and turpentine caught significantly more *D. frontalis* when one of the two traps was baited additionally with either (+)- or racemic *endo*-brevicomin (F=94.6; df=2, 6; P<0.001; Fig. 3). Trap pairs with either (+)- or racemic *endo*-brevicomin did not differ significantly in total catch (Tukey test, P>0.05). Furthermore, the disparity in catch between traps within pairs was significantly altered by the addition of either (+)- or racemic *endo*-brevicomin to one of the traps (F=26.0; df=2,6; P=0.001). Within *endo*-brevicomin-treated trap pairs, the trap that lacked *endo*-brevicomin caught significantly more beetles than its *endo*-brevicomin-baited twin [for (+)-*endo*-brevicomin: t=3.78, df=11, P=0.003; for racemic *endo*-brevicomin: t=3.2, df=11, P=0.008], whereas there was no significant difference in catch between traps within pairs when *endo*-brevicomin was not added to either of the two traps (t=0.86, df=11, P=0.41) (Fig. 3). There were no significant treatment effects on the proportions of females trapped [either total catch per trap pair (F=0.38; df=2,6; P=0.70) or difference between the traps in proportion of females caught (F=0.71; df=2,6; P=0.53)]. The overall proportion of females trapped was 0.42±0.03 (mean ± s.d.).

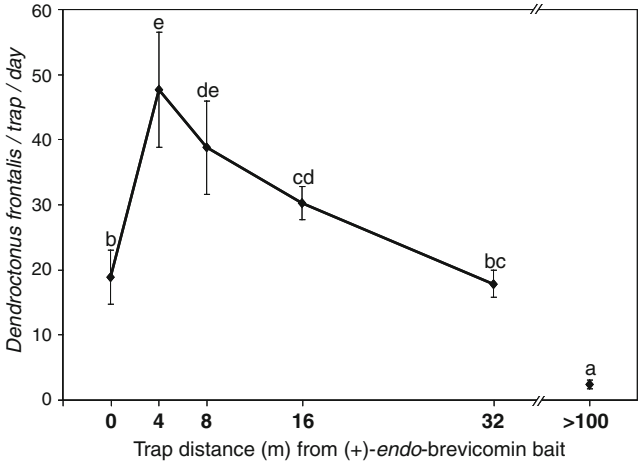

Fig. 2 Experiment 1: Mean (± s.e.) daily catch of *Dendroctonus frontalis* in individual multiple-funnel traps baited with frontalin and turpentine. Traps were positioned >130 m apart, and each trap either had a single release device of (+)-*endo*-brevicomin placed a specified distance (0, 4, 8, 16, 32 m) away from it or had no (+)-*endo*-brevicomin bait assigned [i.e., the closest (+)-*endo*-brevicomin bait was in an adjacent treatment or >100 m away)]. Means associated with the same letter were not significantly different (Tukey test on cube root transformed data, α=0.05). Means and s.e.'s were calculated from the untransformed data with six observations averaged within each Latin square (N=3)

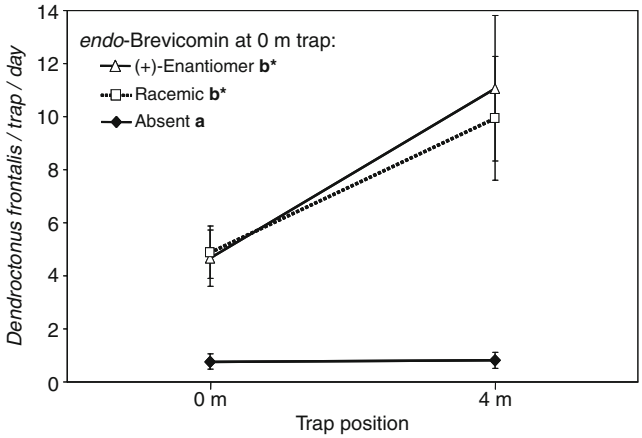

Fig. 3 Experiment 2: Mean (± s.e.) daily catch of *Dendroctonus frontalis* in traps arranged in pairs with 4 m spacing between traps and >100 m spacing among pairs. All traps were baited with frontalin and turpentine, and one randomly-chosen trap per pair (the "0 m trap") was baited additionally with racemic *endo*-brevicomin, (+)-*endo*-brevicomin, or nothing. Treatment names in legend inset associated with the same letter did not differ significantly in summed beetle catch per pair (Tukey test on cube root transformed data, α=0.05); those with an asterisk had a significant difference in catch between the two traps within the pair (paired *t*-test; α=0.05). Means and s.e.'s were calculated from the untransformed data with three observations averaged within each Latin square (N=4)

Experiment 3: Trios of Adjacent Traps with Varying endo-Brevicomin Release Rate Total catch of *D. frontalis* by trios of frontalin/turpentine-baited traps was significantly affected by the rate of (+)-*endo*-brevicomin released from the middle trap (F=34.3; df=3,9; P<0.001; Table 2). The disparity in catch between the outer and middle traps was likewise influenced by the release rate of (+)-*endo*-brevicomin (for X_{4m}-X_{0m}: F=10.3; df=3,9; P=0.003; for X_{1m}-X_{0m}: F=16.9; df=3,9; P<0.001) . In general, increasing the release rate of (+)-*endo*-brevicomin at the middle trap enhanced total catch by the trios, and it caused a relatively greater increase in catch at the two outer traps than at the middle trap (Table 2). Release rate of (+)-*endo*-brevicomin from the middle trap had no effect on the proportions of females trapped [either total catch per trio of traps (F=0.73; df=3,9; P=0.56) or difference between outer and middle traps (F<0. 28; df=3,9; P>0.84)]. The overall proportion of females trapped was 0.48±0.04 (mean ± s.d.).

Experiment 4: Trios of Adjacent Traps with Variable Baits The disparity in catch between the outer and middle traps of trap trios was significantly influenced by altering the bait component that was removed in the outer trap treatments (for X_{4m}-X_{0m}: F=13.0; df=2,4; P=0.018; for X_{1m}-X_{0m}: F=8.41; df=2,4; P=0.037). When (+)-*endo*-brevicomin was absent from the outer traps, catch in each outer trap was greater than in the middle trap, whereas the reverse occurred when frontalin was absent (Table 3). The arrangement of baits had no significant effect on the difference in proportions of females trapped by the outer and middle traps (F<2.36; df=2,4; P>0.21). The overall proportion of females trapped was 0.45±0.06 (mean ± s.d.). When trap trios were established with all three baits at all three traps (Table 3, corollary data set), catch in the outer traps did not differ from catch in the middle trap (for X_{4m}-X_{0m}: t=0.099; df=13; P=0.92; for X_{1m}-X_{0m}: t=0.127; df=13; P=0.90).

Discussion

Horizontal displacement of the release point of a single release rate of the male-produced pheromone component *endo*-brevicomin significantly enhanced its synergistic effect on *D. frontalis* attraction to sources of female-produced frontalin and host odors (experiment 1, Fig. 2). We are not aware of previous reports in which separation of the release points of synergistic insect pheromone components caused enhanced attraction. Incremental separation of a pair of traps each baited with different synergistic components of the aggregation pheromone of the western pine beetle, *Dendroctonus brevicomis* LeConte, caused a rapid, logarithmic decline in attraction of this bark beetle (Byers 1987). For two moth species [cabbage looper, *Trichoplusia ni* (Hübner), and the spotted stem borer, *Chilo partellus* (Swinhoe)], separation of the release points of two sex pheromone components by mere centimeters significantly reduced the percentage of males reaching the sources (Linn and Gaston 1981; Lux et al. 1994).

Of the three major components of the aggregation attractant for *D. frontalis* (i.e., frontalin, (+)-*endo*-brevico-

Table 2 Experiment 3. Effect of varying release rate of (+)-*endo*-brevicomin on catch of *Dendroctonus frontalis* by trios of adjacent funnel traps[a]

Release rate of (+)-*endo*-brevicomin at 0 m trap[b]	Trap position within trio	Catch (No./trap/d)[c]	Total catch (No./trio/d)[c,d]	Difference in catch between 4 m and 0 m trap[c,d]	Difference in catch between 1m and 0 m trap[c,d]
Absent	0 m	0.70±0.35	2.42±1.46a	0.33±0.37ab	0.00±0.05a
	1 m	0.70±0.40			
	4 m	1.03±0.71			
Low	0 m	0.94±0.26	3.11±0.79a	0.22±0.16a	0.08±0.04a
	1 m	1.02±0.29			
	4 m	1.16±0.26			
Medium	0 m	5.02±1.45	19.4±4.71b	2.65±0.46bc	1.68±0.46b
	1 m	6.71±1.74			
	4 m	7.67±1.63			
High	0 m	6.49±1.83	28.7±8.63b	5.90±2.53c	3.30±1.11b
	1 m	9.80±2.88			
	4 m	12.39±4.06			

[a] Three traps were arranged in a straight line with the outer traps located either 1 m or 4 m from the middle ("0 m") trap. All three traps were baited with frontalin and turpentine, and the release rate of (+)-*endo*-brevicomin from the 0 m trap was varied.

[b] See Table 1.

[c] Mean ± s.e. Standard errors were calculated for untransformed data with four observations averaged within each Latin square (N=4).

[d] Means within a column associated with the same letter were not significantly different (Tukey test on cube root transformed data, α=0.05).

Table 3 Experiment 4: Effect of varying bait assignment on catch of *Dendroctonus frontalis* by trios of adjacent funnel traps[a]

Bait component removed from 1 m and 4 m traps[b]	Trap position within trio	Catch (No./trap/d)[c]	Difference in catch between 4 m and 0 m trap[c,d]	Difference in catch between 1 m and 0 m trap [c,d]
Compared treatments[e]				
(+)-*endo*-Brevicomin	0 m	2.98±0.89	2.14±1.13b*	0.76±0.44b*
	1 m	3.73±1.23		
	4 m	5.12±1.91		
Frontalin	0 m	1.54±0.60	−1.40±0.55a*	−1.14±0.44a*
	1 m	0.40±0.16		
	4 m	0.14±0.05		
Turpentine	0 m	1.69±0.39	−0.50±0.34a	−0.07±0.25ab
	1 m	1.62±0.46		
	4 m	1.19±0.33		
Corollary data set[f]				
None (all three baits at all three traps)	0 m	7.24±1.68	0.44±0.81	0.40±0.71
	1 m	7.64±1.96		
	4 m	7.69±2.09		

[a] Three traps were arranged in a straight line with the outer traps located either 1 m or 4 m from the middle ("0 m") trap. The 0 m trap received all three bait components, whereas the 1 and 4 m traps were baited identically and each lacked a single component.

[b] See Table 1.

[c] Mean ± s.e.

[d] Means within a column associated with the same letter were not significantly different (Tukey test on cube root transformed data, $\alpha=0.05$). Asterisk denotes rejection of the hypothesis that the difference equaled zero (one sample *t*-test, df=8, $P<0.05$)

[e] Treatments compared in a Latin square experimental design. Means and s.e.'s calculated from untransformed data with three observations averaged within each Latin square ($N=3$)

[f] Descriptive data set collected separately from 14 trap trios with all three traps baited identically with all three bait components. Data excluded from statistical comparisons among treatments. Means and s.e.'s calculated with each trio as the unit of replication ($N=14$)

min, and host monoterpenes; Sullivan et al. 2007), only (+)-*endo*-brevicomin and frontalin appeared to mediate beetle discrimination among closely-spaced traps (experiment 4, Table 3). Beetle captures were concentrated in traps that were relatively closer to a frontalin source, but relatively more distant from a (+)-*endo*-brevicomin source (Table 3). This tendency of *D. frontalis* to be trapped close to the source of frontalin is consistent with its proposed function as a releaser of landing behavior for this species (Hughes 1976) and likely reflects the general tendency of bark beetle aggregation pheromones to concentrate beetle flight and landings near their release point (Coster and Gara 1968; Tilden et al. 1979; Laidlaw and Wieser 2003). In contrast, *endo*-brevicomin does not appear to direct landings close to its point of release. Although its release rate was varied across three orders of magnitude, catch was never higher in a trap amended with (+)-*endo*-brevicomin than in unamended traps positioned 1 and 4 m distant (experiment 3, Table 2), and the synergistic effect of (+)-*endo*-brevicomin was the same whether the release device was placed directly on a frontalin/turpentine-baited trap or 32 m away from it (Fig. 2). Thus, *endo*-brevicomin appears to function as a synergist on an area-wide scale, enhancing attraction and landing of beetles to any sources of frontalin/host odors within a radius of tens of meters.

With the exception of the highest release rate used in experiment 3, the *endo*-brevicomin release rates in our experiments (Table 1) were within the range that we estimate is produced by a single pine undergoing mass attack by *D. frontalis*. Based on calculations and data reported in Coulson et al. (1976) and Fargo et al. (1978), approximately $0.4–1.8\times10^3$ *D. frontalis* pairs per day infest a 25–40 cm diameter loblolly pine in the first 4 d following initiation of mass attack, whereas an individual male *D. frontalis* paired for <1 d with a female produces 291±55 ng *endo*-brevicomin in an 18±2 hr period (Sullivan et al. 2007). Thus, during the first days of a mass attack, we conservatively estimate that a single mass-attacked tree yields 0.12–0.52 mg *endo*-brevicomin/d. Our estimate is also in agreement with that of Browne et al. (1979) for the release rate of frontalin (the male-produced bicyclic acetal aggregation pheromone component of the sibling species, *D. brevicomis*). These authors reported the collection of 0.4 and 3.3 μg frontalin per m of bole per hr from two different infested ponderosa pines. When extrapolated over 24 hr and 5 m of infested bole their measurement represents a release rate of 0.048 and 0.40 mg/d/tree. Since stand densities recommended for host species of *D. frontalis* produce an average 4–10 m spacing among trees of susceptible ages (Nebeker et al. 1985), we deduce that *endo*-brevicomin

arising from a single infested tree could synergize attraction of beetles to attacks on potentially dozens or hundreds of neighboring trees.

Additionally, at an adequately high release of *endo*-brevicomin, this synergy would evidently be stronger at adjacent trees than the tree of pheromone origin. Thus, elevated (+)-*endo*-brevicomin concentrations associated with high densities of beetle pairs should disproportionately enhance beetle responses to neighboring trees that are in the early stages of colonization by solitary, frontalin-releasing females. Pheromones are believed to play an important role in shifting the focus of bark beetle attack from fully-colonized trees to neighboring trees that are either in the initial stages of colonization or not yet attacked (Renwick and Vité 1970; Borden 1989). Furthermore, synchronization of such host "switching" by the beetle population is evidently critical to the sustained growth of *D. frontalis* infestations (Gara and Coster 1968). Several authors have proposed that host switching in *D. frontalis* and other bark beetles is mediated by the compounded effects of pheromones of opposing activities: long-range attractants from both the old and new attack foci draw beetles into the area while simultaneously short-range inhibitors deter landings on old attack foci (Johnson and Coster 1978; Payne 1980; Berryman 1982; Schlyter et al. 1987a). In contrast, *endo*-brevicomin may promote host switching by *D. frontalis* through a single principle, namely, the disproportionate enhancement of attraction to relatively more distant sources of frontalin/host odors.

Bark beetle semiochemicals have been classified as either synergists or inhibitors typically with "attractant-challenge"-type experiments in which a bait of undetermined activity is attached to a randomly-selected, attractant-baited trap, and catch is compared to a simultaneously-operated, attractant-only control trap (Reeve and Strom 2004; Fettig et al. 2006). If catch in the challenged trap is significantly lower than the control, the test bait is concluded to be inhibitory; if catch is significantly higher than the control, the test bait is concluded to be a synergist or attraction enhancer (Payne et al. 1978a; Bedard et al. 1980; Bakke 1981; Pureswaran and Borden 2004; Sullivan 2005). Our data demonstrate how such attractant-challenge tests might generate misleading or inconsistent conclusions regarding the activity of a compound. In experiment 2 (Fig. 3), catch in the randomly chosen, *endo*-brevicomin-challenged trap of a pair was significantly lower than in its 4 m-distant, attractant-only baited twin (the latter being equivalent to a control in an attractant-challenge type experiment employing 4 m trap spacing). Thus, according to the traditional interpretation of such data, *endo*-brevicomin inhibited catch at the experimental trap. However, since catch by both traps of the pair was much lower in the absence of *endo*-brevicomin, the difference within the pair was apparently due to greater enhancement of catch in the control trap than the challenged trap and not to inhibition in the challenged trap.

Theoretically, any synergist possessing the property displayed by *endo*-brevicomin in experiment 1 (i.e., maximizing beetle response to an attractant when displaced a short distance from it, what we shall hereafter call "displacement-enhanced synergism") could be interpreted in a challenge test to be either a synergist or an inhibitor depending solely upon the trap spacing used (Fig. 4). This is because challenge experiments employing a simultaneously-operated control trap cannot distinguish whether the test bait alters catch in the challenged trap rather than (or simultaneously with) the control trap. The ability to conclude enhancement or inhibitory activity in such tests relies on the assumption that traps are adequately spaced to assure that they cannot influence one another. However, the minimum separation required to assure trap independence is difficult to determine experimentally and has rarely been sought in research on bark beetle flight behavior (Dodds and Ross 2002). It should be noted that many of the interpretations of the results from the present study rely on the assumption that the minimum 100 m distance between adjacent experimental subjects (i.e., individual traps in experiment 1, pairs in experiment 2, and trios in experiments 3–4) was adequately large to prevent mutual interference.

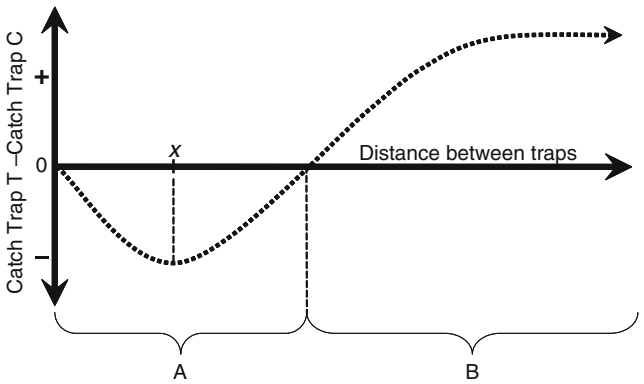

Fig. 4 Hypothetical responses of insects to two adjacent traps baited identically with an attractant where one trap chosen at random (treatment trap, "trap T") is baited additionally with a compound possessing the properties exhibited for (+)-*endo*-brevicomin in experiment 1 (Fig. 2), namely, it has (1) synergistic activity when attached directly to the trap and (2) maximum synergistic activity when displaced distance *x* from the trap (i.e., displacement-enhanced synergism). The opposite trap ("trap C") is baited only with the attractant, and is thus equivalent to the control trap in a classic "attractant-challenge"-type trapping bioassay (see text). The Y-axis represents the arithmetic difference in catch between the two traps; the X-axis represents the distance between the two traps. Within distance range A, catch is greater in the control trap than the trap holding the experimental bait, and thus the "apparent" effect of the bait is inhibition. However, at greater distances (range B), catch is less in the control trap than the experimental trap, and the apparent effect of the bait is enhancement

We suspect that interference among adjacent experimental traps compounded with *endo*-brevicomin's capacity for displacement-enhanced synergism may explain why many earlier studies failed to detect *endo*-brevicomin's synergistic activity. Intertrap spacing of less than 30 m is typical for studies evaluating bark beetle semiochemicals (Payne et al. 1978b; Bedard et al. 1980; Bakke 1981; Borden et al. 1987; Miller and Borden 1992; Sullivan 2005) and was 25 and 15–20 m in the only two trapping studies that both observed *endo*-brevicomin inhibition for *D. frontalis* and reported these experimental parameters (Payne et al. 1978a; Salom et al. 1992). Catch in the control traps of these tests was possibly enhanced by the nearby *endo*-brevicomin baits (i.e., attached to experimental traps) leading to the conclusion that the *endo*-brevicomin was reducing catch in the experimental trap to which it was attached. Experiment 1 demonstrated that *endo*-brevicomin can have synergistic effects on attractant-baited traps as far as 32 m away, hence greater trap spacing than this would be necessary to assure absence of intertrap effects. The two aforementioned studies were also performed within the limits of an active *D. frontalis* infestation, and *endo*-brevicomin released from naturally-infested trees within these infestations could likewise have altered responses to traps. One highly significant implication of our results is the possibility that additional bark beetle species possess pheromone components whose attractive activity has been overlooked due to the use of experimental procedures that concealed this activity.

Vité et al. (1985) demonstrated that the antipodes of *endo*-brevicomin elicited conflicting behavioral responses from *D. frontalis*, and they hypothesized that the inhibitory activity reported for *endo*-brevicomin in previous studies was due to the use of high release, racemic baits in which the antagonistic (–)-enantiomer overwhelmed the attractive (+)-enantiomer. In our experiment 2, baits consisting either of two capillaries of racemic *endo*-brevicomin or of a single capillary of (+)-*endo*-brevicomin [and thus having an identical dose of (+)-*endo*-brevicomin but a differing dose of (–)] exhibited essentially identical activity (experiment 2, Fig. 3). Thus, the (–)-enantiomer did not diminish the synergistic activity of the (+)-enantiomer at the release rates that we tested. Rather, experiment 2 demonstrated that both pure (+) and racemic baits could induce greater catches in more distant traps, and thus either might appear to behave as an attractant synergist or inhibitor under appropriate experimental conditions (Fig. 4).

The mechanism that underlies displacement-enhanced synergism likely involves the concentration of the relevant components in the behavioral chemical plume, and displacement of a modifier compound (i.e., a synergist or an inhibitor) from an attractant-baited trap should influence pheromone concentrations within the plume downwind of the trap in predictable ways. These include: 1) the average concentration of the modifier within the attractant plume will be reduced, causing the active space of the combination (i.e., the zone in which both the attractant and modifier exceed the response threshold of the insect) to be smaller; 2) the relative proportions of the attractant and modifier will vary greatly within the overlapping portions of the plumes; and 3) the concentration of modifier at the attractant-baited trap and close to it will drop to zero unless the modifier is released directly upwind from the attractant (adapted from Byers 1987). Thus, reducing the modifier's release rate should at least partially duplicate the effects of displacing the modifier, particularly regarding effects 1 and 3 above. In support of this inference, spatial displacement of a modifier (either a synergist or an inhibitor) from the release point of an insect semiochemical generally produces the same outcome as reducing the dose of or eliminating the modifier: displacement of inhibitors increases (McLaughlin et al. 1974; Witzgall and Priesner 1991; Liu and Haynes 1992; Rumbo et al. 1993; Potting et al. 1999), whereas displacement of synergists reduces insect responses to a semiochemical (Linn and Gaston 1981; Byers 1987; Lux et al. 1994). However, in experiment 3, reducing the dose of (+)-*endo*-brevicomin at the center trap caused a reduction in *D. frontalis* catches at both the center and the adjacent traps, not an increase in catch at the center trap as would be predicted if the relatively higher catch in the outer traps was attributable simply to a relatively lower concentration of (+)-*endo*-brevicomin at these locations. Thus our data are not consistent with the hypothesis of an entirely dose-driven mechanism for the displacement-enhanced synergism of *endo*-brevicomin.

In addition to synergists and inhibitors, bark beetles may also utilize "multifunctional" semiochemicals that are attractive or synergistic at low release rates but inhibitory at high ones (Rudinsky 1973; Borden et al. 1987; Schlyter et al. 1987a; Seybold et al. 1992; Erbilgin et al. 2003). Furthermore, bark beetle responses to certain attractive semiochemicals decline at very high release rates (Seybold et al. 1992; Ross and Daterman 1998; Miller et al. 2005). Semiochemicals possessing such a parabolic dose-response curve should possess a single, optimally-attractive release rate; therefore the attractive activity of a bait exceeding this optimum dose could conceivably be enhanced by displacing it from the target (e.g., a trap), thereby effectively reducing its dose both at and downwind of the target. Additional experiments would be necessary to determine whether the (+)-enantiomer of *endo*-brevicomin produced by *D. frontalis* (Sullivan et al. 2007) has the parabolic dose-response of a multifunctional pheromone, however evidence suggests that at least racemic *endo*-brevicomin possesses this dynamic (Vité et al. 1985). Results of previous studies suggest that elevated doses of multifunctional pheromones may enhance insect attraction to or landing at distant locations. When

Rabaglia and Lanier (1983) increased the rate that the multifunctional pheromone α-multistriatin was released from the bole of host trees, landings by the European elm bark beetle, *Scolytus multistriatus* (Marsham), switched from the bole to the crown. Elevating the release rate of the multifunctional pheromone ipsdienol at a trap baited with aggregation pheromone increased the relative proportion of *Ips typographus* (L.) trapped in 3 m-distant, unbaited traps while it increased the summed catch for all traps (Schlyter et al. 1987b). The possibility that displacement-enhanced synergism is a common property of multifunctional semiochemicals is worthy of further investigation. Enhanced knowledge of semiochemical plume dynamics in heterogeneous environments integrated with data from further studies that vary both the release rate and displacement of attraction modifiers will help to elucidate the mechanism underlying displacement-enhanced synergism.

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the lack of temperature effects on pheromone production can be found (e.g., Pandey and Pandey 1990). Certain insects exhibit pheromone-releasing behaviors that increase in frequency with increasing temperature, leading to periodicity in pheromone release that can be both circadian and seasonally controlled (e.g., Sower et al. 1971; Pope et al. 1982). In all vertebrates studied to date with described or putative pheromones, pheromone production and expression are thought to be controlled almost exclusively by hormones (e.g., amphibians, Yamamoto et al. 1996; Iwata et al. 2000a; Kikuyama et al. 2005; reptiles, Mendonça and Crews 1996; birds, Rajchard 2007; rodents, Bruce 1965; Thiessen et al. 1968; Mugford and Nowell 1971; primates, Michael 1975; goats, Iwata et al. 2000b). However, in at least one vertebrate, the red-bellied newt, *Cynops pyrrhogaster*, pheromone production may be promoted by low temperature (Iwata et al. 2000a; Takahashi et al. 2001).

Our focal species, the red-sided garter snake, *Thamnophis sirtalis parietalis*, is a reptilian model for understanding the relationship between seasonality, behavior, and sex pheromones. After a prolonged winter dormancy (8 mo, Gregory 1976), these animals emerge from limestone hibernacula by the thousands in the interlake region of Manitoba, Canada. At this time, males vigorously court and compete for females, leading to large mating balls of males courting single females (Aleksiuk and Gregory 1974). Courtship behavior in male red-sided garter snakes is affected by changes in temperature, with prolonged, low temperature dormancy (12 wk, 4°C) enabling robust, stereotypical courtship behavior in the laboratory after simulated emergence from hibernation (Hawley and Aleksiuk 1975; Garstka et al. 1982). Because the snakes hibernate several meters underground, there is no light, and photoperiodic cues have been shown to have no effect on the induction of spring mating behavior (Whittier et al. 1987; Lutterschmidt et al. 2006). Instead, the one seasonal signal, low temperature dormancy, causes changes in aromatase activity in the sexually dimorphic nuclei of the brain of males (hypothalamic pre-optic area, HPOA; Krohmer et al. 2002). The HPOA hypertrophies by the time of emergence, and is the neural center for the control of courtship behavior in this species (Krohmer et al. 2002). Laboratory-simulated hibernation conditions induce changes in the secretion patterns of melatonin in males, suggesting that this hormone may play a role in the regulation of courtship behavior as well (Lutterschmidt 2006). Thus, male red-sided garter snakes have evolved a behavioral response to a specific, relevant environmental cue: temperature (Crews and Moore 1986).

Female red-sided garter snakes exhibit seasonal changes in two components of female reproduction: receptivity and attractivity (Beach 1976). Sexual receptivity is controlled primarily by estrogen, with ovariectomy abolishing receptivity and estrogen replacement in castrated females reinstating receptivity (Crews 1976). Attractivity is determined by using bioassays of male courtship behavior, and both the quantity and quality of the female sexual attractiveness pheromone blend can be determined easily with chemical analyses (Mason et al. 1989, 1990). The female sex pheromone of the red-sided garter snake is a series of nonvolatile, long-chain (C_{29}–C_{37}) saturated and monounsaturated methyl ketones ranging from 394 to 532 Da (Mason et al. 1989). Individual pheromone components, when presented singly, elicit much lower levels of courtship from males compared to the complete blend; however, the longest, unsaturated components can elicit significant courtship behavior when presented alone (Mason et al. 1989). More recent work has shown that the ratio of the abundances of unsaturated to saturated components relays information about female reproductive condition. Long and/or fat females produce more offspring and elicit more vigorous courtship from males when compared to females of lesser condition (LeMaster and Mason 2002; Shine et al. 2003). Sex pheromone blends from large females consist primarily of the long-chain, unsaturated components, specifically those with masses of 476, 504, and 532 Da, whereas sex pheromone blends from small females are composed equally of saturated and unsaturated methyl ketones that span the entire range (394-532 Da; LeMaster and Mason 2002). Males prefer isolated pheromone extracts from large females compared to small females, suggesting that they choose females based solely on differences in the composition of the pheromone, namely the ratio of unsaturated to saturated components (LeMaster and Mason 2002; Shine et al. 2003). Thus, the female sex pheromone is an honest signal that relays information about reproductive condition.

Early laboratory studies on garter snakes have shown that males display increased interest in females during the time of shedding (as evidenced by increased tongue-flicking and chin-rubbing behavior), and this may be attributed to changes in the quantity of the pheromone produced by the skin (Noble 1937; Kubie et al. 1978). The quality of the pheromone differs between the breeding (spring) and nonbreeding (fall) seasons in the red-sided garter snake, suggesting that the pheromone relays information about season as well as sex (Mason et al. 1987; LeMaster and Mason 2001). However, pheromone profiles for this species are complex: there are at least 16 unique methyl ketone molecules comprising a single female pheromone profile (Mason et al. 1989). To fully understand this complexity, analyses are needed of pheromone profiles from individual red-sided garter snakes, however, this was not done in the previous two studies on pheromone seasonality in this species (Mason et al. 1987; LeMaster and Mason 2001).

The purpose of this study was to examine changes in the pheromone profiles from individual female garter snakes that may be affected by the process of hibernation. Initiation of sexual behavior in males is dependent on exposure to prolonged low temperature, and it may be that female attractivity also is affected by the low temperature dormancy that this species experiences annually. In the field, it is impossible to gather pheromone samples from females during hibernation because they are several meters below ground (Lutterschmidt et al. 2006). Thus, our laboratory study utilized simulated hibernation to determine how both the quantity and quality of the female sexual attractiveness pheromone may change during winter dormancy.

Methods and Materials

Animals Female red-sided garter snakes, *Thamnophis sirtalis parietalis,* (N=24) were captured at the hibernaculum in the fall of 2005 (Inwood, Manitoba, Canada). Pheromones were extracted from subsets of these snakes (N=8 each) at three different times: fall, winter (during hibernation), and spring. The fall sample was collected at the hibernaculum, whereas the remaining snakes were transported back to the laboratory at Oregon State University and placed into artificial hibernation approximating natural conditions (4°C; 0:24 h L:D; 85% RH). Pheromones were collected from the winter group after 12 wk in winter hibernation (January), and those for the spring group were collected 12 wk later (April) after 1 wk of simulated emergence (12°C, 10 h:6°C, 14 h, L:D). All procedures involving the use of live animals were approved by the Institutional Animal Care and Use Committee at Oregon State University (ACUP 3120) and were in compliance with guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The collection and use of these animals was approved by Manitoba Conservation (Manitoba Wildlife Scientific Permit WB02024).

Pheromone Collection and Isolation We followed previously published methods for collection and analysis of pheromone extracts (Mason et al. 1989). Snakes were sacrificed with a lethal overdose of brevital sodium (6 mg/kg) prior to collection of skin lipids from individual snakes by immersion in hexane for 12 h. The snakes were then removed from the solvent, and snake mass (g), snout-vent length (SVL, cm), and midbody circumference (cm) were recorded. The volume of the skin lipid extracts was reduced under vacuum with a rotary evaporator, and the total skin lipid yield of the dry product was determined (mg) before fractionation. The pheromone was isolated by using alumina columns [activity III (Sigma-Aldrich, St. Louis, MO, USA); pooled fractions 4-6 (2% diethyl ether:98% hexane as mobile phase)]. The pooled fractions containing pheromone were reduced to dryness by using a rotary evaporator and weighed to determine the mass (mg; termed "pheromone fraction mass" hereafter). The combined pheromone fractions are composed almost solely of female sex pheromone methyl ketones (>99% of fraction mass) and have been demonstrated to elicit courtship behavior from males that is as intense as courtship directed to live females in the den in the spring (Mason et al. 1989). The pooled pheromone fractions were resuspended in a pheromone:hexane mixture (1 mg:1 ml) before analysis by gas chromatography-mass spectrometry.

Gas Chromatography-Mass Spectrometry Individual pheromone samples were analyzed with a Hewlett Packard 5890 Series II gas chromatograph fitted with a split injector (280°C) and an HP 5971 Series mass selective detector. Aliquots (1 µl) of the 1:1 samples (1 mg pheromone:1 ml hexane) were injected onto the fused-silica capillary column (RTX-1; 15 m×0.25 mm i.d., 0.25 µm film thickness; Restek Corporation, Bellefonte, PA, USA) with helium as the carrier gas (5 cm/sec). All injections were made in the splitless mode with the split valve closed for 60 sec. Oven temperature was held initially 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 held at 310°C for 5 min. Individual compounds were identified by using mass spectral data and ion chromatograms comparing our spectra to published data and authentic standards (Mason et al. 1990). By using the peak integration function in ChemStation software (Agilent) interfaced with the GC-MS, we determined relative contributions of each component of the pheromone to the overall profile of each snake.

Data Processing and Statistical Analysis We tested for global differences in total skin lipid mass, pheromone fraction mass, pheromone concentration, unsaturated to saturated component ratio, individual unsaturated and saturated component mass, and low and high molecular weight contributions to profiles by using one-way and two-way ANOVAs (time, component type as factors) followed by pairwise comparisons (Tukey Tests; SigmaStat v.3.1). Total skin lipid mass and pheromone fraction mass were arcsine transformed after correcting for snake mass before analyses. Pheromone fraction mass was used to derive pheromone concentration (µg/cm^2) by using previously published methods and log-transformed before analysis (Mason et al. 1990; LeMaster and Mason 2002). Briefly, the circumference (cm) at midbody was measured and then multiplied by the snout-to-vent length (cm) to get total surface area (cm^2), and pheromone fraction mass (mg) was converted to µg and divided by total surface area to yield pheromone concentration (µg/cm^2). By using an internal

standard (methyl stearate, 10 μg/ml hexane; LeMaster et al. 2008), we were able to derive individual component mass (μg) for all of the 16 methyl ketones comprising the pheromone. Global differences in pheromone structure were analyzed by using the Multi-Response Permutation Procedure in the vegan package for R (v.1.8-8; McCune et al. 2002). Pairwise comparisons for pheromone structure were run by using the same procedure but by excluding a new group each time. Coordinates for a non-metric multidimensional scaling plot to represent differences in individual pheromone profiles also were generated with the vegan package, and all graphics were created in SigmaPlot (v.8.0).

Results

Total skin lipid and pheromone fraction masses increased from fall to spring ($F_{2,\ 21}$=95.471, P< 0.001; $F_{2,\ 21}$=9.196, P= 0.001; Fig. 1). Total skin lipid mass was higher in both spring and winter than in fall (q= 17.893, P< 0.001; q= 15.750, P< 0.001, respectively). Pheromone fraction mass was higher in spring than in fall (q= 6.065, P= 0.001). No other differences were found in skin lipid or pheromone fraction masses. Pheromone concentration (μg pheromone/cm^2 of skin) also increased over time (H=14.791, P< 0.001), with the pheromone concentration being higher in spring and winter than in fall (q=4.850, P=0.005; q= 4.525, P=0.006, respectively; Fig. 2).

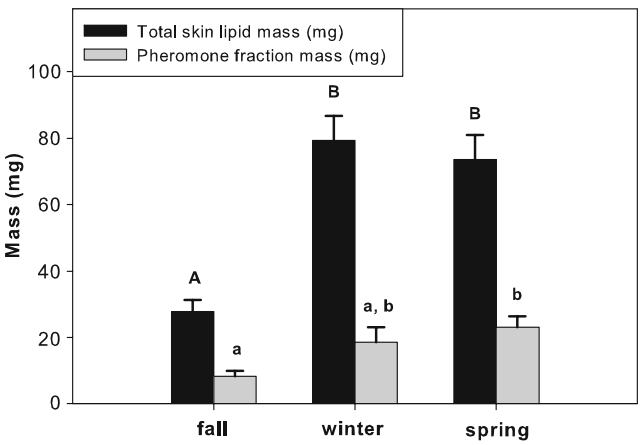

Fig. 1 Change in mass (mg; mean + s.e.; N=8 for each bar) of both total skin lipids (black bars) and pooled fractions containing only the nonvolatile methyl ketones that comprise the pheromone (pheromone fraction mass; gray bars) of female red-sided garter snakes. Pheromones were collected from individual females in the fall at the snake den, during winter in laboratory-simulated hibernation, and in the spring in the laboratory after simulated emergence. Different letters of the same case (e.g., "a" vs. "b") represent significant differences (P< 0.05) in mass for those sampling periods

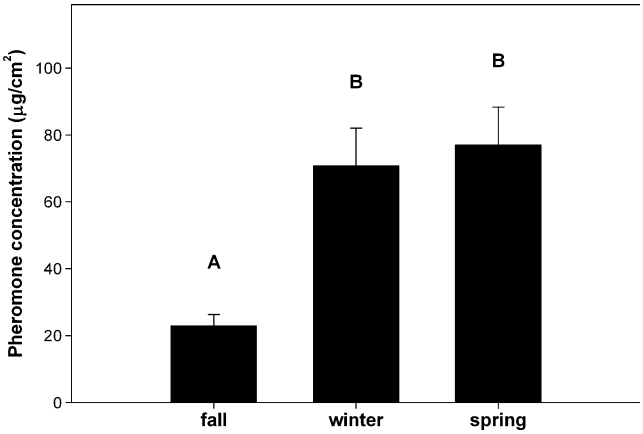

Fig. 2 Sex pheromone concentration (μg pheromone/cm^2 skin; mean + s.e.; N=8 for each bar) extracted from female red-sided garter snakes in the field and during laboratory-simulated hibernation and emergence. Different letters represent significant differences (P<0.05) in pheromone concentration for those sampling periods

Following the GC-MS analysis, we found that the composition of the pheromone blends changed over time (A= 0.07672, P= 0.016). Spring profiles were different than fall profiles (A= 0.1089, P= 0.012), but no other differences were significant (Fig. 3). We then tested the pheromone profile characteristics that may have contributed to these global differences. We found no difference in the ratios of the abundances of unsaturated to saturated components within the pheromone profiles over time ($F_{2,\ 18}$=0.301, P> 0.05). Both main effects (season, component type [saturated, unsaturated]) were significant when we analyzed the masses of pheromone components ($F_{2,\ 47}$=8.703, P< 0.001; $F_{1,47}$=18.329, P< 0.001, respectively). There was no season x component type interaction ($F_{2,\ 47}$=2.010, P= 0.147). Within season, the mass of unsaturated components was greater than the mass of saturated components for fall (q=3.004, P=0.04), winter (q= 3.772, P=0.011), and spring (q= 3.218, P=0.028; Fig. 4). Within component type, the mass of saturated components was greatest in spring compared to fall (q=4.561, P=0.007), but no other differences were detected. The mass of unsaturated components was greater in spring than in fall (q= 4.774, P= 0.005), and winter than in fall (q= 3.706, P=0.032; Fig. 4). The compounds contributing most to the overall makeup of the pheromone blend were the 504 (unsaturated), 476 (unsaturated), 450 (saturated), and 448 (unsaturated) Da molecules (Fig. 3).

Components within each pheromone profile were arbitrarily split into two molecular weight classes: low molecular weight (LMW; <463 Da) and high molecular weight (HMW; >463 Da). Previous work has demonstrated that the attractivity of female red-sided garter snakes increases with increasing molecular weight and unsaturation of individual components of the pheromone, suggest-

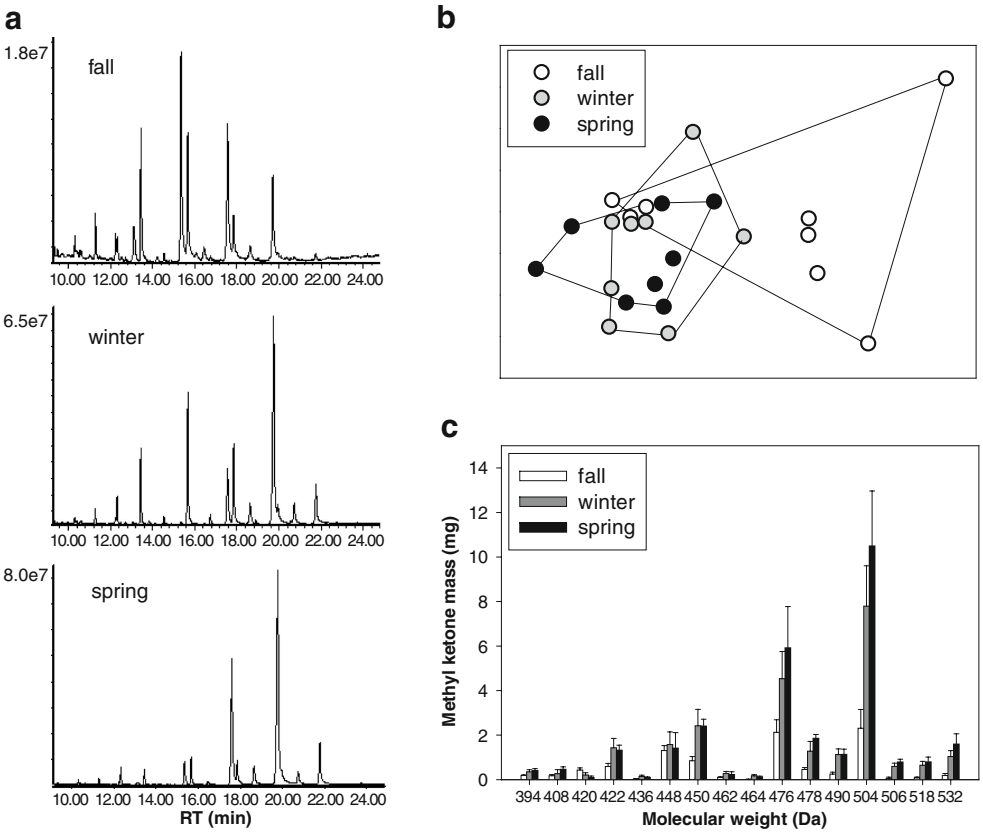

Fig. 3 Changes in the female sexual attractiveness pheromone blend of red-sided garter snakes from three sampling periods (fall, winter, spring). **a** Representative total ion chromatograms of sex pheromone blends from individual females from the three sampling periods. **b** Non-metric multidimensional scaling plot showing individual pheromone profiles. Minimal convex polygons are drawn for each group to show how the area of similarity for pheromone profiles shifts and contracts from fall to spring, with individual profiles being most similar (most tightly clustered) in spring. The fall (white circles) and spring (black circles) were significantly different ($P<0.05$). **c** Masses (mg; mean + s.e.; $N=8$ for each bar) of prospective individual components (categorized by molecular mass; Da) of the female sex pheromone blend over the three sampling periods. The most abundant compounds were the 504 (unsaturated), 476 (unsaturated), 450 (saturated), and 448 (unsaturated) Da methyl ketones

ing that changes observed in the molecular weight of pheromone constituents over time have biological consequences in nature (Mason et al. 1989). Thus, we analyzed the ratio of HMW:LMW proportions over time (log-transformed) and found a significant time effect ($F_{2,\ 21}=5.362$, $P=0.013$). Pheromone profiles were dominated by HMW compounds in spring compared to fall ($q=4.365$, $P=0.015$), but no other differences were significant (winter vs. fall, $q=3.522$, $P>0.05$).

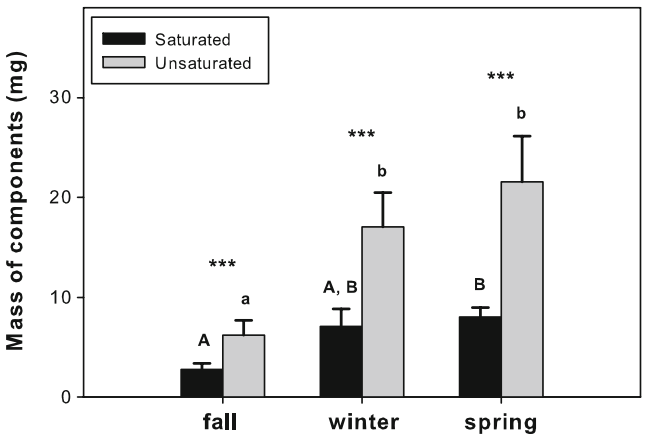

Fig. 4 Mass of components (mg; mean + s.e. ; $N=8$ for each bar) comprising the female sex pheromone blend of red-sided garter snakes from three sampling periods (fall, winter, spring) grouped by methyl ketone type (saturated, unsaturated). At all three sampling periods, unsaturated components were significantly more abundant ($P<0.05$; asterisks) than saturated components, especially in the spring. Within each class, both types of components reached peak mass in the spring. Different letters of the same case represent significant differences ($P<0.05$) within a class

Discussion

We have shown that the process of hibernation induces quantitative and qualitative changes in the female sexual attractiveness pheromone of the red-sided garter snake. Both total skin lipid mass and pheromone fraction mass increased from fall to spring as did the concentration of pheromone present on the skin. The quality of the pheromone also appeared to change concomitant with the changes in amount and concentration, with females producing pheromones dominated by unsaturated, high molecular weight (HMW) methyl ketones by the winter and into spring. Thus, hibernation is critical in the regulation of one of the two components of female reproduction in this species: attractivity.

Temperature has numerous effects on sexual signals, both in vertebrates and invertebrates. For instance, ambient temperature affects the length and color of the manes of lions, an information-rich sexual signal (West and Packer

2002). Vertebrates and invertebrates exhibit fluctuating asymmetry in sexually selected traits that reflect changes in ambient temperature and photoperiod (e.g., tail length in swallows, Møller and Szep 2005; sex combs in flies, Polak and Starmer 2005). Further, many animals use sex-specific sounds to attract and evaluate mates, and both the production and perception of these signals can be altered significantly by changes in temperature (e.g., flies, Ritchie et al. 2001; frogs, Gerhardt 1978; Shimizu and Barth 1996). Our results suggest that a powerful sexual signal, the female attractiveness pheromone of garter snakes, changes significantly as a result of low temperature dormancy (i.e., hibernation).

Sex pheromone production is known to be affected by temperature in red-bellied newts, *Cynops pyrrhogaster*, where low temperatures (8–12°C) induce increased synthesis of prolactin mRNA, and prolactin, in conjunction with androgens, can induce hypertrophy of the secretory capacity of the newt pheromone (sodefrin) gland and stimulate production of sodefrin (Toyoda et al. 1994; Yamamoto et al. 1996; Iwata et al. 2000a; Takahashi et al. 2001). Sodefrin is a peptide pheromone, thus the mechanisms controlling its synthesis and expression will differ from those acting in garter snakes to produce the relatively small methyl ketone molecules that act as pheromones in our system. However, both systems are responsive, at least in part, to changes in temperature, and they warrant further study. Our study is the first in a reptile to demonstrate the effects of hibernation on female attractivity at the level of pheromone production.

Female receptivity and male courtship behavior in both amphibians and reptiles are known to be affected by changes in temperature. Low temperatures are critical for either initiating or maintaining expression of both male courtship and female receptivity in a number of salamanders, newts, and frogs (Duellman and Trueb 1994). In reptiles, the effects of temperature on sexual behavior in males have been studied more extensively than in females (Whittier and Tokarz 1992). Male red-sided garter snakes do not express typical courtship behavior in the spring unless they experience an extended (8–17 wk) low temperature dormancy (4°C, 0 h:24 h L:D; Camazine et al. 1980; Garstka et al. 1982). Female receptivity in garter snakes is influenced by low temperature dormancy, but hormonal priming (estrogen) before hibernation exerts a much stronger effect, as evidenced by abolished receptivity in ovariectomized females and only reduced receptivity resulting from exposure to warmer temperatures during winter dormancy (Bona-Gallo and Licht 1983; Mendonça and Crews 1996). Our results suggest that female attractivity is optimized by low temperature dormancy, though our study did not include a warm hibernation control group due to the high rate of female mortality demonstrated in previous studies (16%, Whittier et al. 1987).

Collectively, research on this system has shown that maximal, coordinated sexual behavior in the red-sided garter snake (male courtship and female attractivity and receptivity) is contingent upon sustained, low temperature dormancy. This species overwinters in large, communal dens, and snakes emerge en masse in the tens of thousands to engage in spectacular displays of scramble mating as males search for and court singly-emerging females (Aleksiuk and Gregory 1974; Gregory 1976). In an evolutionary sense, this species has adapted to maximally express sexual characteristics (behavior, pheromones) at a time when population densities are highest and precision in identifying and selecting mates is critical.

The role of skin and gland-derived compounds as pheromones in sauropsids (birds and reptiles) may have arisen from the modification of phospholipids originally derived for waterproofing the integument (adapted from Maderson 1986). In another sauropsid (mallard, *Anas platyrhynchos*), the waterproofing gland (uropygial) produces sex pheromones, and pheromone synthesis occurs from lysosomal modification (lengthening, esterification) of monoester waxes, and ultimately is regulated by female sex steroid hormones (Jacob et al. 1979; Kolattukudy and Rogers 1987; Bohnet et al. 1991). Thus, skin waxes generated primarily for waterproofing have been co-opted into a secondary role as sexual signals in this species. The pheromone of red-sided garter snakes may be similarly derived. First, the skin may increase its lipid production to retard transcutaneous water loss during low temperature dormancy, which is a common process in sauropsids during cold acclimation (e.g., turtles, Willard et al. 2000; pigeons, Peltonen et al. 2000). Second, as is the case in mallards, the lipids would be modified by enzymes that are activated by cold temperatures. The data presented in this paper show that the pheromone of female red-sided garter snakes becomes dominated by longer, unsaturated molecules, the most sexually attractive components of the female pheromone, as a result of low temperature dormancy (Mason et al. 1989; LeMaster and Mason 2002; Shine et al. 2003). Several enzymes are activated by cold temperatures in vertebrates that either synthesize or lengthen long-chain lipids (C16 or greater; Jakobsson et al. 2005, 2006). Other enzymes, such as desaturases, play a critical role in maintenance of the phospholipid bilayer by desaturating lipid chains to increase membrane fluidity and prevent damage from freezing (reviewed in Hazel and Williams 1990; Tiku et al. 1996). Such temperature-induced mechanisms (elongation, desaturation) may exist in the pheromone synthesis of red-sided garter snakes and warrant further study.

We have shown that female pheromone content is altered during hibernation in this species, and that the pheromone present at emergence is composed primarily of long-chain,

unsaturated methyl ketones, which in other studies have been shown to elicit the strongest behavioral response from males (Mason et al. 1989; LeMaster and Mason 2002; Shine et al. 2003). The red-sided garter snake serves as a robust model for testing hypotheses on the seasonality of sexual signal production, and it also represents a point of evolutionary divergence in vertebrate sexual signals. Rather than plumage and pelage to reflect sex, condition and season, the skin lipids of the female red-sided garter snake are modified to signal condition and fecundity so as to be optimally honest at a time when mate density is highest and the odds of mating are virtually certain. In the future, we plan to examine skin lipid production by male garter snakes to determine if they may also display the same seasonal pattern we have shown in females of this species.

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other signals such as pH. However, the diversity of sources of CO_2 in the rhizosphere and the imperfect correlation between regions of the root with high CO_2 and those with highest attraction suggest that CO_2 may not be a specific signal (Bird 1960). In the soil, CO_2 efflux is the result of root and rhizospheric respiration and organic matter decomposition. *Meloidogyne incognita* is repelled by ammonia and several nitrogenous salts released from decaying material (Castro et al. 1991) possibly further modulating its response. For the free-living nematode *Caenorhabditis elegans*, CO_2 has been described both as an attractant and a repellant (Dusenbery 1983; Bretscher et al. 2008; Hallem and Sternberg 2008). Recent studies with *C. elegans* suggest that the degree of CO_2 avoidance depends on strain and nutritional status (Hallem and Sternberg 2008).

Pluronic F-127 (PF-127) has been used by our group to study plant-nematode interactions and nematode behavior (Wang et al. 2009). A 23% PF-127 solution forms a semisolid gel at room temperature but is liquid at 15°C and below. The gel is highly transparent and generally has low toxicity or is non-toxic, depending on the organism and gel concentration used (Gardener and Jones 1984; Ko and Van Gundy 1988). Nematodes can be mixed in PF-127 solution at 15°C. As the solution warms to room temperature, nematodes become suspended in the gel. They can move freely in 3 dimensions through the gel, allowing examination of behavior in response to various treatments. Nematodes dispersed in PF-127 gel are highly attracted to roots, aggregating mainly at the zone of elongation (Wang et al. 2009). In the absence of roots, nematodes aggregate into tight balls containing most of the nematodes that were distributed in PF-127 gel. We have postulated that this aggregation is due to a preference for low oxygen concentration, a response to anhydrobiotic stress, and/or inter-nematode chemical signals (pheromones). The ability to form aggregates differs among RKN species and strains (Wang et al. 2009; Wang and Williamson, unpublished).

Here, we tested the utility of PF-127 gels for examining root-knot nematode movement in gradients of pH and CO_2. Because the genetic map and genome sequence for *Meloidogyne hapla* strain VW9 are available (Opperman et al. 2008), thus opening the potential to explore genes involved in signal recognition and behavior by the nematode, we focused on this nematode in the current work. We have, however, compared the behavior to isolates of other agronomically important species, including *M. incognita*, for which the genome sequence is also available (Abad et al. 2008).

Methods and Materials

Nematodes and Materials *Meloidogyne hapla* strains used were VW9 (Liu and Williamson 2006), NCS (from C. Opperman, North Carolina State University) and LM (from P. Roberts, University of California, Riverside; Chen and Roberts 2003). *M. javanica* strains VW4 and VW5 are a pair of nearly isogenic strains that differ in ability to reproduce on tomato bearing the resistance gene *Mi-1* (Gleason et al. 2008). *M. incognita* strains used were 557R (Yaghoobi et al. 2005), W1(inbred from nematodes isolated from a Woodland, CA tomato field), Beltran (P. Roberts), Harmony (H. Ferris, University of California, Davis), and VW6 (inbred from a field isolate obtained by H. Ferris). All *M. hapla* strains, *M. javanica* strain VW5, and *M. incognita* strains 557R and W1 were maintained on tomato cultivar VFNT, which carries the gene *Mi-1*. The remaining strains were maintained on the nematode-susceptible tomato cultivar UC82. Eggs were collected and axenic J2s produced as described by Branch et al. (2004) with minor modifications (Wang et al. 2009), then rinsed four times with sterile water; larvae hatched at room temperature.

Methanesulfonic acid and pyridine, both 99%, were from Acros Organics (Geel, Belgium). Bromocresol purple was the sodium salt from National Analine Division, New York, NY, U.S.A.. Other chemicals were A.C.S. grade.

Gel Preparation and Attraction Assay PF-127 (Sigma, St. Louis, MO, U.S.A.) solution was 23% w/v in 10 mM Tris, 10 mM morpholino-ethanesulfonic acid (MES), pH 7.1, unless otherwise indicated. Suspended PF-127 was dissolved by stirring for 24 h at 4°C (Wang et al. 2009). Where noted, the pH indicator bromocresol purple was included. The solution was stored at 15°C. Twenty ml of PF-127 solution containing freshly-hatched J2 (6000 J2 per plate unless otherwise indicated) were poured into each Petri dish (nominal 100×15 mm) at 15°C.

"Chemical dispensers" were prepared by cutting standard 5 cm, 200 µl pipette tips (USA Scientific Inc.) 5 mm from the small end and 20 mm from the large end. The test chemical solutions were prepared in 23% PF-127 and maintained on ice. Approximately 100 µl of the test solution were pipetted into each chemical dispenser, which was held horizontally while the solution was allowed to gel at room temperature. The Petri dish with the PF-127 solution and dispersed nematodes was placed at room temperature, and two dispensers were placed anti-parallel with ends not closer than 3 cm from the Petri plate edge, before the gel formed and before bubbles could develop at the dispenser ends. Dispensers rested on the bottom of the plate, and the top of the large opening of the dispenser was approximately at the surface of the gel in the Petri dish when 20 ml of PF-127 solution were used (i.e., 3.4 mm deep gel). At the indicated time after initiation of the assay, the number of nematodes was counted within the 5 mm diam. circle centered at the small end of pipette tip. Control dispensers containing 23% PF-127 in water were used as checks. The

different sizes of the two dispenser openings allowed us to examine two different concentration gradients simultaneously, and the paired dispensers in each plate provided duplicate analyses. At least three plates were included in each experiment, and each experiment was repeated at least twice. Micrographs were captured with a Nikon SMZ-U dissecting microscope by using SimplePCI High Performance Imaging System (Compix Inc, Sewickley, PA, USA). To obtain a broader view of the nematode distribution patterns in response to chemical gradients, lower magnification photographs of Petri plates were taken with a digital camera by using edge lighting and against a black background.

pH Measurements The pH in gels was measured with a pH Meter (Denver Instrument Company, Arvada, CO, USA), by direct insertion into gel of a PHR-146 Micro Combination pH electrode (Lazar Research Laboratories, Inc., Los Angeles, CA, USA). pH meter calibration was performed by the two point standardization method (pH 7 and pH 4) according to the manufacturer's instructions. pH measurements were repeated at least twice.

Carbon Dioxide Delivery Assay A Petri dish lid was modified for delivery of CO_2 to PF-127 gel (Fig. 1). CO_2 sublimed from dry ice in a container was passed via tubing through water and delivered to a central 1.5 cm diam. circle on the gel surface. Water-saturated air was delivered to the remainder of the gel (Fig. 1), thus creating a concentric gradient of CO_2 in air in the gel from the 1.5 cm circle to the edge of the plate.

Statistical Analysis JMP software (SAS Institute Inc., Cary, NC, USA) was used for statistical analyses. Data were subjected to one-way analysis of variance (one way–ANOVA). Results are reported as significant or non-significant in Tukey's Honestly Significant Difference (Tukey HSD) Test ($P<0.05$).

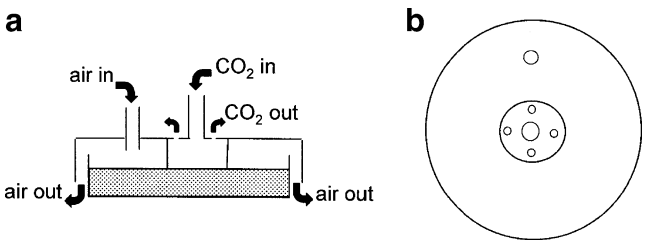

Fig. 1 Apparatus for CO_2 delivery to PF-127 gel. The lid of a standard Petri dish was modified with entry and exit ports for CO_2, and an entry port for air. Air could escape under the edge of the lid. An acrylic cylinder cemented under the lid just touched gel surface to maintain an area of water-saturated CO_2 on the gel surface. The apparatus is shown in cross section (**A**) and top view (**B**). The stippled region in A represents the gel with the nematodes

Results

Formation of pH Gradients in Gel To assess gradient formation in PF-127 gel, the pH indicator bromocresol purple was incorporated into gel in 10 mM Tris-MES buffer, pH 7.1. Two dispensers with 0.17 M acetic acid were placed into each plate. After 4 h, yellow circles were seen around the openings of the dispenser, indicating that proton diffusion occurred in a radial pattern from the openings of the dispenser. The size of the circles increased slowly over the next 20 h (Fig. 2).

Aggregation of M. hapla (Strain VW9) in pH Gradients The response of *M. hapla* strain VW9 J2 was monitored in pH gradients produced around the ends of dispensers that had been charged with 0.17, 0.34, or 0.85 M acetic acid. Nematode clustering was observed at the dispenser small end 3 h (not shown) after insertion and was maintained after 5 and 24 h (Fig. 3A and B, respectively). Five h after insertion, the gradient near the dispenser large end extended over a sufficient gel volume so that J2 responded by congregating into a halo (Fig. 3C, 0.34 M, and 0.85 M acetic acid dispensers). Presumably, the halo was generated because J2 prefer a specific low pH range to the even lower pH near the dispenser end and the pH 7.1 of the PF-127 gel in which J2 were originally suspended. Figure 4 presents J2 density related to distance from the dispenser large end for 0.34 M and 1.7 M acetic acid. The observed distributions conformed to a normal curve, and the gradient created by 0.34 M acetic acid stimulated J2 to achieve a density ten times greater than the initial density of J2 in the gel at the most dense part of the halo (Fig. 4).

By using a microelectrode, pH was measured at the boundaries of the halo of high nematode density. For both low pH and high pH boundaries, more than 50 readings were made from three plates. At 7 h after assay initiation, the pH was 4.54±0.05 for the inner boundary and 5.40±0.15 for the outer boundary. At 20 h after assay initiation, the circle was larger, but the pH values at the boundaries were similar (pH 4.58±0.17 for inner boundary and pH 5.37±0.13 for outer boundary). The experiment was repeated, and the same boundary pH values were obtained. No aggregation of nematodes was observed with any of the checks that contained dispensers with 23% PF-127 in water (not shown).

To test whether acetate from ionized acetic acid was sufficient to account for gradient-directed nematode migration, the response of *M. hapla* J2 was observed for dispensers with 10 and 50 mM sodium acetate, corresponding to the range of acetate ion expected in the previously tested acetic acid-loaded dispensers. At 24 h, no nematode aggregation was seen at the openings of the dispensers (results not shown), suggesting that protons or

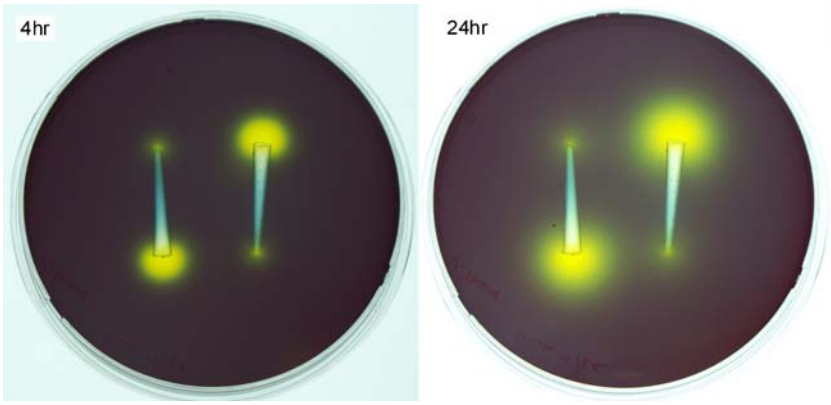

Fig. 2 pH gradient formation in PF-127 gel. The pH indicator bromocresol purple (pH 5.2–6.8, yellow to purple) was included in the gel. The dispensers initially contained 0.17 M acetic acid, and plates are shown 4 and 24 h after the dispenser was inserted into the gel

acetic acid rather than acetate ions affected the nematode aggregation.

By using the dispenser system, we tested the response of VW9 to a variety of Brønsted (proton-donating) acids, including strong acids (HCl, H_2SO_4, $HClO_4$, methanesulfonic acid), carboxylic acids (acetic, citric, formic, lactic, propionic, and succinic), pyridinium hydrochloride, and pyridinium methanesulfonate. Strong acids and three

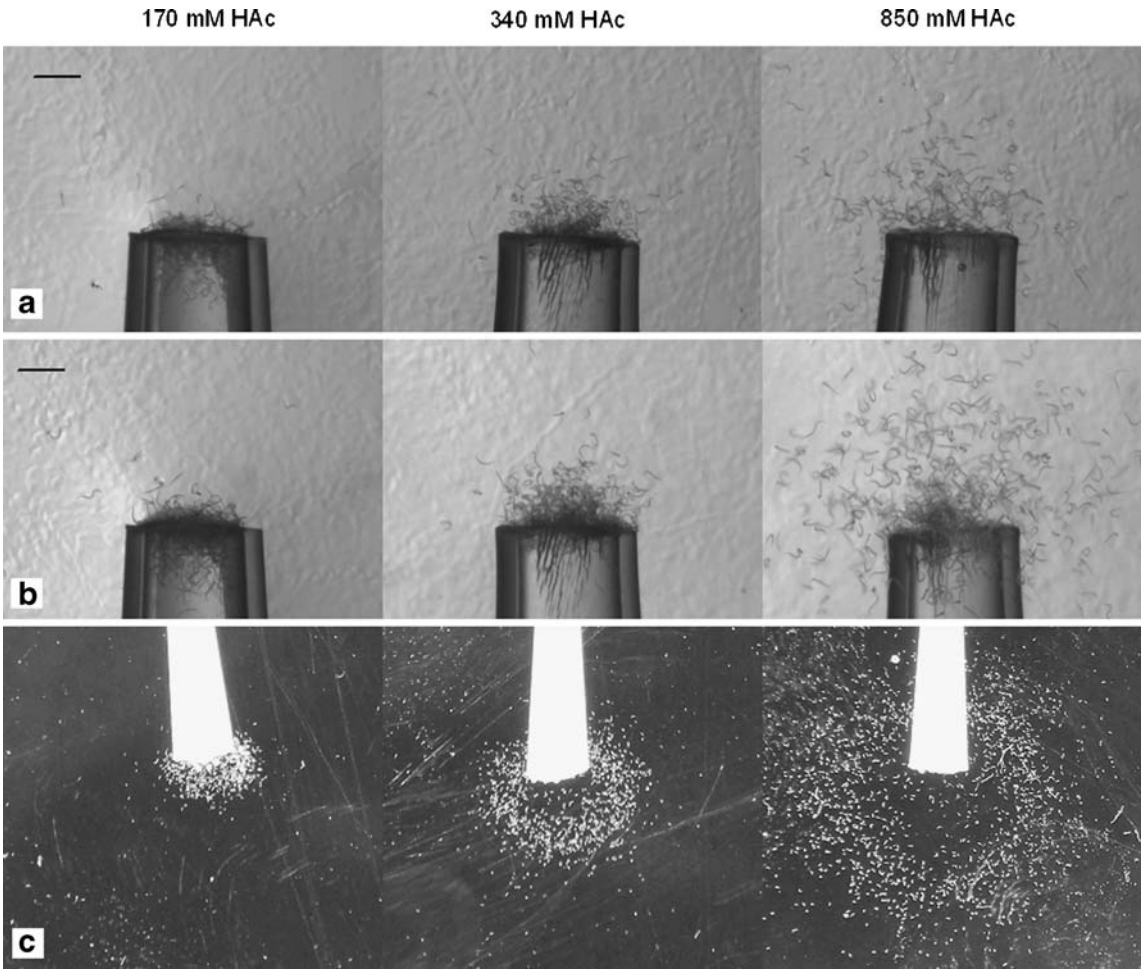

Fig. 3 Migration of *Meloidogyne hapla* VW9 J2 in acetic acid gradients in PF-127 gel. Dispensers contain 0.17 M, 0.34 M, or 0.85 M acetic acid as indicated at the top of the three columns of images. Dissecting microscope images of the dispenser small end were recorded at 5 h (**A**) and 24 h (**B**) after inserting dispensers in the gel. Scale bar = 1 mm. Lower magnification edge-lighted photographs of the dispenser large end at 24 h is shown in C. The diameter of the large end of the dispenser is~4 mm

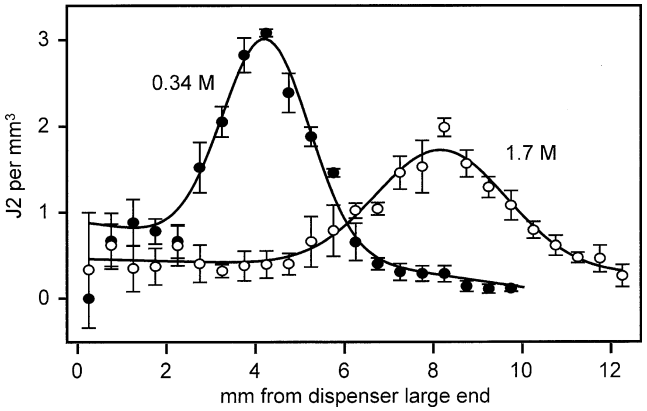

Fig. 4 Distribution of VW9 J2 in acetic acid gradients. Dispensers containing acetic acid in 23% PF-127 gel were immersed in buffered PF-127 gel containing 0.30 J2 per mm^3. After 5 h, the gel was photographed with edge lighting. Ninety degree arcs, scaled to 0.5 mm wide in the original gel, were superimposed on the enlarged photographic images with the origin of the arcs at the middle of the dispenser wide end. J2 within each arc were counted for the 0.34 M (●) and 1.7 M (○) acetic acid dispensers (four dispensers from three plates), and the data were transformed to J2 per mm^3 of gel. The average values and standard deviations are presented against distance from the dispenser large end. The standard deviation expanded at small distances from the dispenser because of the small volumes corresponding to the short arcs. Data were fit to Gaussian curves by a least-squares method

monoprotic carboxylic acids (acetic, formic, and propionic acids) induced movement of the nematodes to form a halo centered at the wide opening of the dispenser (Figs. 3C and 5A–C) and aggregation at the small opening (Figs. 3A&B, and 5G). For lactic, citric, and succinic acids, the J2 migrated to and aggregated inside the dispensers (for example, see Fig. 5E and H). The ability of citric acid and citrates to act as a J2 attractant was tested by charging dispensers with 0.40 M citrate plus citric acid buffers at pH~4.5 or ~5.5 (corresponding the observed pH values at the halo edges) and with sodium, potassium, ethanolammonium, or diethanolammonium as the cation. All of these citrate buffers, like citric acid itself, attracted J2 well into the dispensers, rather than just to the openings, suggesting that citrate/citric acid attracted J2 sufficiently to overcome any tendency to form a halo in a citric acid gradient (results not shown). Pyridinium ions were selected as another Brønsted acid because of the similarity of the pK_a (5.2) to that of acetic acid (4.8). Pyridinium methosulfonate (Fig. 5E) and pyridinium chloride (not shown) also caused aggregation of J2 inside the tip in a manner similar to that seen for citric acid.

Root-knot Nematode Species and/or Strains Differ in Responsiveness to Acetic Acid Different species and strains of root-knot nematodes differ in the rate at which they aggregated at root tips in PF-127 gel (Wang et al. 2009). Responses of different RKN species and strains to acetic acid gradients were compared 3 h after initiation of the assay (Table 1). *Meloidogyne hapla* strain VW9 had the highest aggregation at the dispenser tip of all the RKN strains tested. The nearly isogenic *M. javanica* strains VW4 and VW5, which differ in their ability to parasitize tomato with the resistance gene *Mi-1*, did not show significant differences in attraction to acetic acid. However, significant differences were seen among *M. incognita* strains derived from isolates originating from different locations and different hosts. Strains VW6, Beltran and W1 aggregated significantly less than *M. incognita* strain 577R. *M. incognita* strain Harmony, which was originally isolated from grape, aggregated much more slowly than the other strains. By 24 h and for several species and strains, the number of nematodes near the dispenser tip was too great to count with confidence, and was scored as described in Fig. 6. However, even for strain Harmony, the number of nematodes at the tip increased between 3 and 24 h, and the relative aggregation of the different strains remained the same (Table 1). The pH range of maximum aggregation was assessed at 24 h by using a microelectrode, and all were found to aggregate between pH 4.5 and 5.4. Thus, it did not appear that the strains differed in the pH range to which they were attracted, but they did differ in the rate at which they congregated in the gel region of that pH range.

Response of Nematodes to Gel Exposed to CO_2 To investigate RKN J2 response to CO_2, we delivered CO_2 to a 1.5 cm circle in the center of a PF-127 gel plate buffered with 10 mM Tris-MES, pH 7.1, in which J2 of *M. hapla* VW9 were distributed evenly. In a parallel experiment, CO_2 was delivered to a plate with the same gel, but without nematodes and with the pH indicator bromocresol purple. By two hours, the region of the gel exposed to CO_2 had acidified sufficiently to turn the indicator yellow (i.e., pH 5.2 or less), and nematodes began to aggregate outside the border of the circle. At 24 h, the yellow area remained limited to the circle area exposed to CO_2 with a gradient to blue in the 3 mm just outside the circle. Nematodes aggregated outside the circle area (Fig. 7A) whereas nematode numbers and distribution within the circle exposed to CO_2 did not noticeably change during the 24 h. The pH of the CO_2 exposed area was measured as 5.0–5.3, depending on position tested, and the pH outside the circle area was about 6.0. When delivery of CO_2 was stopped after 24 h, and the plate was examined at 48 h, nematodes were found to have aggregated within the circular area. At this time, the pH inside the circle was around 5.6, whereas that outside was around 6.0. This experiment was repeated four times with similar results. In a shorter time course, delivery of CO_2 was stopped after 4 h, and plates were observed at 24 h. In this experiment also, nematodes aggregated around the circle while CO_2

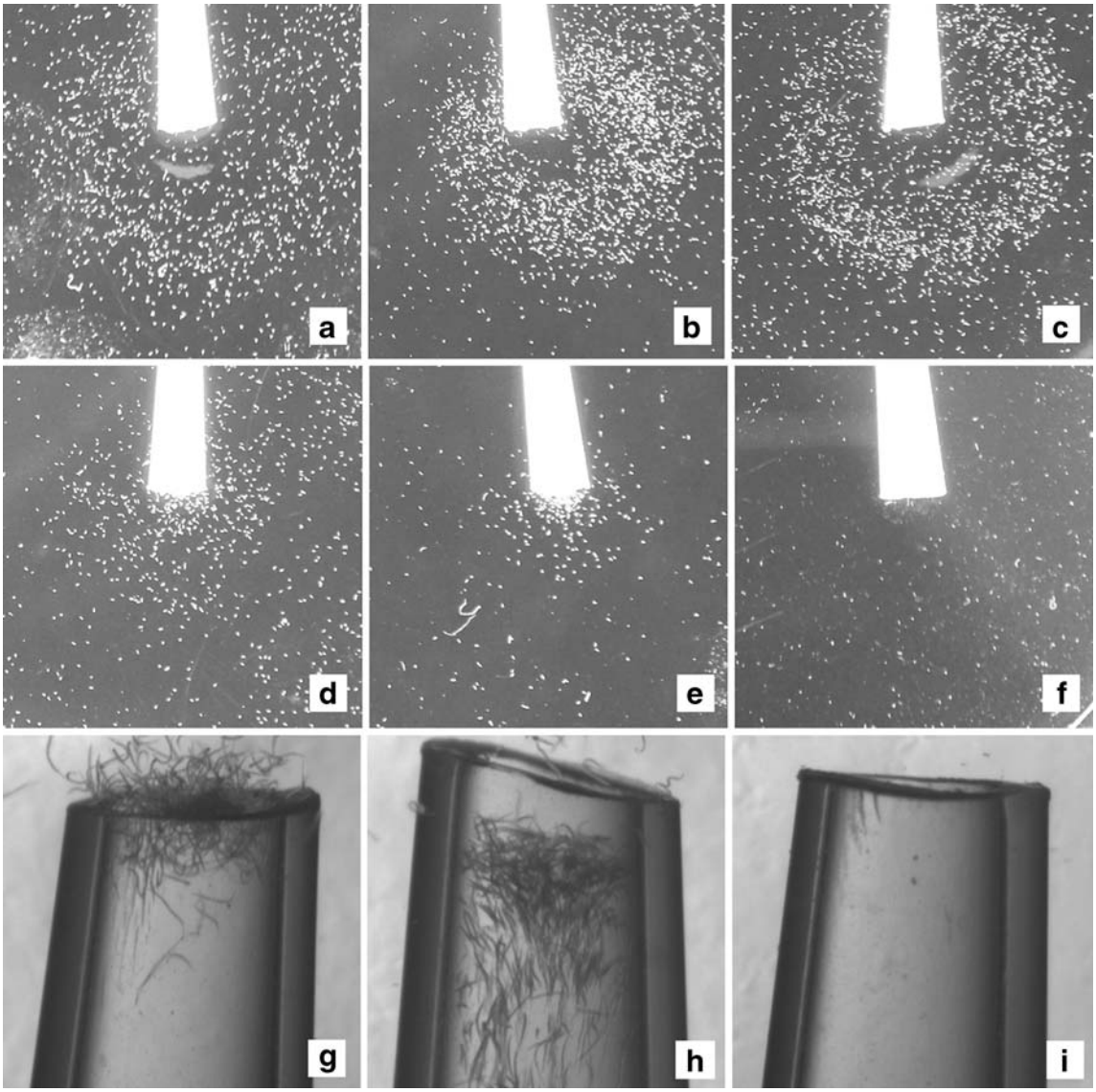

Fig. 5 Response of *Meloidogyne hapla* VW9 J2 to acid gradients in PF-127 gel. Each assay was in a Petri dish containing 6000 J2 in 20 ml of gel in 10 mM Tris-MES, pH7. Dispensers contained 0.40 M solutions of HCl (**A**), propionic acid (**B**, **G**), methane sulfonic acid (**C**), or pyridinium methanesulfonic acid (**E**), or 0.16 M citric acid (**D**, **H**), or water (**F**,**I**). All photographs were taken at 20 h after insertion of the dispensers. Panels A-F show the large end of the dispenser and were taken with edge lighting against a black background. Panels G-I show small opening of dispenser, and photomicrographs were taken under a dissecting microscope

was delivered, then moved inside during the next 24 h after the source of CO_2 was disconnected. At the latter time the pH inside the circle was ~5.8 whereas that outside was ~6.1.

To distinguish the response to CO_2 from that of reduced pH, the CO_2 experiment was repeated with gel buffered with 10 mM sodium acetate, pH 4.9. At 24 h after continuous CO_2 exposure, nematodes remained uniformly distributed throughout the gel and did not aggregate at the border of the circle (Fig. 7B). Using a microelectrode, the pH inside the circle was determined to be approximately 4.7 and that in the surrounding gel 4.9. At 24 h after removal of the CO_2 source, nematodes aggregated within the circle but to a much lesser extent than when the gel was buffered at pH 7.0.

Discussion

All tested strains of RKN J2 aggregated in a gel zone with a pH range of 4.5–5.4, e.g., from an acetic acid gradient. These species, as well as many other plant pathogens/symbionts, are strongly attracted to and invade roots in the zone of elongation. Growing root cells extrude protons, with the elongation zone being the most acidic region (Mulkey and Evans 1981; Pilet et

Table 1 Attraction to acetic acid of species and strains of root-knot nematode[a]

Species/ Strain	Isolate host and location	Nematodes within 5mm of tip	
		3h	24h
Meloidogyne hapla			
VW9	Tomato, CA	106.8A	+++
NCS	Unknown, NC	62.8B	++
LM	Unknown, France	71.5B	++
M. javanica			
VW4	Unknown, CA	57.0BC	+
VW5	Unknown, CA	50.5C	+
M. incognita			
VW6	Cotton, CA	26.8D	57A
Beltran	Lima bean, CA	37.0D	73A
Harmony	Grape, CA	4.5E	30.5B
557R	Tomato, NC	58.8BC	++
W1	Tomato, CA	28.8D	81.0A

[a] Two chemical dispensers filled with 0.17 M acetic acid were used for each plate and three plates for each treatment. At the indicated time after insertion of the dispensers, the number of nematodes within the 5 mm diam. circle, centered at the small end of pipette tip, was counted. The counts were corrected by subtracting similarly counted nematode numbers for dispensers containing gel alone. Significant differences between counts on different nematode strains at the 3 h time point are indicated with letters A-E ($P<0.05$). Nematodes continued to aggregate so that counts were not possible at 24 h for some trials, but relative nematode densities were assigned symbols +, ++, and +++ following the examples in Fig. 6. For the four strains whose numbers were assessed at the 24 h time point, significant differences are indicated by letters A and B ($P<0.05$).

al. 1983; Peters and Felle 1999). Based on pH indicator dyes, this zone was found to be less than pH 5 for maize seedlings grown on agar plates (Mulkey and Evans 1981). Using PF-127 gel and pH indicator dyes, we also found that *Medicago truncatula* and tomato seedlings produced an acid gradient around the root and could acidify the medium to pH 5 or less immediately adjacent to the root (Wang and Williamson, unpublished). The correlation of the most acidic region of the root with that which is most attractive to the nematodes is consistent with the possibility that pH gradients are signals that direct nematodes to the elongation zone of growing roots. However, while low pH is a marker for the growth zone of a wide range of host plants, the influence of the growth zone on pH in the soil is likely much less than it is in water or gels (Nichol and Silk 2001). In addition, we found that while the preferred pH range was the same for all root-knot nematode species tested, the rate of aggregation within the zone of preferred pH varied with the species and strains of root-knot nematode. *Meloidogyne hapla* VW9 aggregated most rapidly, whereas all *M. incognita* strains, except 577R, were significantly slower to aggregate than *M. javanica* and *M. hapla* species. These results cannot be reconciled directly with our previous observations of the relative migration rates of these same nematode species to host roots; i.e., movement of *M. incognita* and *M. javanica* J2 to tomato roots was much more rapid than that of *M. hapla* (Wang et al. 2009). Thus, pH is probably not the sole attractant to roots, and its relative importance may depend on the nematode strain as well as the host.

Fig. 6 Scoring aggregation of root-knot nematodes to acetic acid. The small opening of the dispenser with 170 mM acetic acid is shown at 24 h after initiation of the assay. **A**. *M. javanica* strain VW4. This sample would be scored as "+" in Table 1. **B.** *M. hapla* strain LM. This sample was scored as "++." **C.** *M. hapla* VW9 was scored as "+++." Scale bar = 1 mm

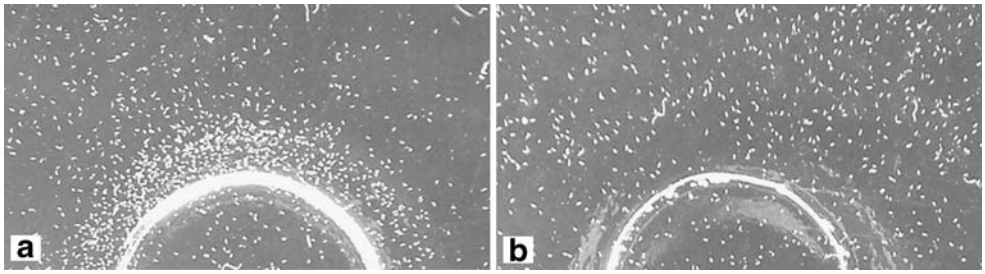

Fig. 7 Response of *Meloidogyne hapla* VW9 to CO_2 in PF-127 gel. **A**. At 16 h after continuous exposure to CO_2, nematodes aggregated just outside the area exposed to CO_2 with pluronic gel buffered with 10 mM Tris-MES, pH 7. **B**. No aggregation was observed when gel was buffered with sodium acetate, pH 4.9. At the start of the assay, 9000 J2 were dispersed in 30 ml PF-127 and poured into the Petri dish. The images show an arc of the 1.5-cm diameter circle that was exposed to CO_2. Note that the number of J2 inside the circle is similar in A and B

While all the acid solutions that we tested resulted in nematode aggregation, some appeared to be more attractive than others. For example, for citric, lactic, and succinic acids, nematodes migrated into the dispensers rather than forming a halo around the tip, suggesting that these acids may be specific attractants. In support of this, citrate salts with various cations at pH values of 4.5 or 5.5 also caused the nematodes to aggregate within the tip. Pyridinium hydrochloride and pyridinium methane sulfonate were tested because these Brønsted acids are similar to the tested carboxy acids in their acid gradient forming potential. However, the pyridinium salts proved to be strong attractants to RKN J2s; in agreement with this observation, pyridine has been reported to be an attractant for *C. elegans* (Dusenbery 1976). It is likely that nematodes respond to and integrate complex signals and/or combinations of signals in their natural environment and that perceived gradients may act in a synergistic or antagonistic manner. The assay described here has potential to identify and dissect some of these interactions.

Carbon dioxide has been widely reported to be an attractant to free-living, insect-parasitic and plant-parasitic nematodes (Pline and Dusenbery 1987; Robinson 1995; O'Halloran and Burnell 2003). However, some of these studies were carried out without buffers, and carbon dioxide can lower pH of aqueous solutions. In our assays, nematodes were attracted to just outside the CO_2-permeated central cylinder (Fig. 1) when the gel was in 10 mM Tris-MES, pH 7.1 (Fig. 7A), whereas nematodes remained uniformly distributed throughout when the gel was buffered with 10 mM sodium acetate, pH 4.9 (Fig. 7B). A pH value of 4.9 was selected because it is within the pH 4.5 to 5.4 range observed for the halos of nematodes that formed in acetic acid gradients (Fig. 3C) and because the calculated pH for water saturated with CO_2 is 4.4. That is, when the initial pH is 4.9, little perturbation of the gel pH is expected from introduction of CO_2; our gel pH measurements agreed with this expectation. Thus, for the pH 7.0 gel assay, it is likely that what attracted J2 to the region around the central cylinder was a pH gradient, not CO_2.

For either pH 7.0 or 4.9, the number of nematodes in the central cylinder of gel did not change in number while the cylinder was continuously exposed to CO_2. However, nematodes entered this area after the delivery of CO_2 was terminated. There are several possible explanations for the failure of the nematodes to enter the CO_2-exposed region. CO_2 is an anesthetic, and concentrations of CO_2 above 10% have been reported to cause a decrease in movement for *M. incognita* (Pline and Dusenbery 1987). Thus, perhaps the nematodes regained ability to move into the central region once the level of the dissolved gas had decreased sufficiently. Our pH measurements indicate that the pH remained lower in the central region compared to the periphery, possibly contributing to the attraction. In addition, it is likely that the oxygen level was lower in the region exposed to CO_2, and root knot nematodes may prefer less than ambient oxygen levels as does *C. elegans* (Gray et al. 2004). As with any in vitro assay, caution should be used in extrapolating in vitro experiments with more complex soil conditions. However, this system has the potential to provide new insights into nematode behavior in natural soil.

The method of dispenser immersion in PF-127 gel described here is simple in design and execution, and it produced steady-state pH gradients in which nematode movement was easy to visualize and document over time. Results were reproducible enough in both nematode distribution and maximum density, so that data from several plates could be combined and presented as a close approximation to a normal distribution of J2 density vs. distance from the dispenser end (Fig. 4). This system has advantages over agarose-based methods in which the test chemical is applied to a well in the agar (Wuyts et al. 2006) or on the surface of the gel (Ward 1973; Matsuura et al. 2007) to form a gradient. For agar-based assays, the volume of test substance that can be applied is limited, and sometimes a second application is needed to maintain the

gradient, whereas with our system the dispensers provided an ample reservoir of test chemical. The PF-127 assay medium in which nematodes are dispersed is three-dimensional. Therefore, the nematode is not constrained to orientations that may be sub-optimal for perception of chemical gradients by amphids and can move in any direction in response to a detected gradient. Photographs with edge lighting allowed population scale analyses of nematode behavior in response to chemical stimuli, although nematode entry into the dispensers was obscured. Under the microscope, local gatherings of nematodes, in the dispenser and elsewhere, were observed, and the clarity of the PF-127 gel allowed the examination of the behavior of individual nematodes.

Assays of this type have great potential for chemotaxis-response studies with a variety of potentially attracting substances, including root tissues (Wang et al. 2009) or small organisms, and should be suited for the study of the responses of a variety of small invertebrates, fungal zoospores, and other subjects.

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Introduction

Papyriferic acid (PA; Fig. 1) is a dammarane triterpene and the principal component of the resin secreted by glandular trichomes (resin glands) produced by the current-annual-growth (CAG) twigs of the juvenile ontogenetic phase (seedlings and saplings; Kozlowski 1971) of the resinous tree birches *Betula neoalaskana* (synonym *B. resinifera*) and *B. pendula* (Reichardt 1981; Reichardt et al. 1984; Lapinjoki et al. 1991; Taipale et al. 1994; Bryant and Julkunen-Tiitto 1995; Julkunen-Tiitto et al. 1996). Several observations indicate that PA deters browsing by mammals. The intensity of browsing by the mountain hare (*Lepus timidus*; Rousi et al. 1991; Tahvanainen et al. 1991) and by the field vole (*Microtus agrestis*; Pusenius et al. 2002) on juvenile *B. pendula* correlated negatively with the density of resin glands on CAG twigs. Moose (*Alces alces*) foraging in birch provenance gardens maintained by the British Columbia Ministry of Forests severely browsed the seedlings and saplings of *B. papyrifera* but only lightly browsed the seedlings and saplings of *B. pendula* growing within a few meters (M. Carlson per. comm.). The juvenile ontogenetic phase of *B. papyrifera* produces only a trace of PA or no PA, whereas juvenile *B. pendula* produces abundant PA (Julkunen-Tiitto et al. 1996; Bryant et al. 2009). By using feeding trials with captive snowshoe hares (*L. americanus*), Reichardt et al. (1984) verified PA deterrence for this important browser of the North American boreal forest.

Reichardt et al. (1984) also demonstrated that captive snowshoe hares cannot meet their daily per capita browse requirement and maintain weight when restricted to a diet of PA-rich browse. When fed the PA-rich winter-dormant CAG twigs of *B. neoalaskana* saplings, the hares' maximum dry mass intake was 38 g/hare/d. When it is not extremely cold, the dry mass requirement for hares is about 150 g/d (Bookhout 1965), increasing as temperature declines (Pease et al. 1979) to a maximum of about 200 g/d at extremely low temperatures (Bryant unpublished data). PA toxicity is the most logical explanation for the starvation-level intake by hares of the PA-rich birch browse.

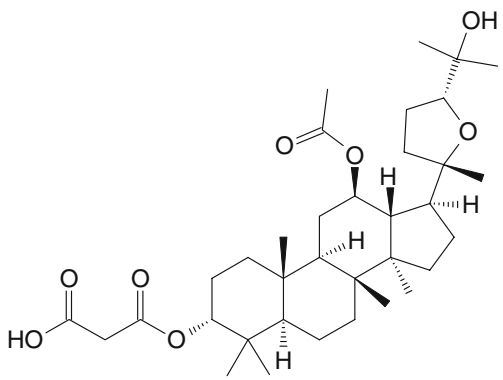


Fig. 1 Structure of papyriferic acid

This study aimed to characterize the biological effects of PA that underly its toxicity and resultant antifeedant effect in mammals. Papyriferic acid is a derivative of the triol, betulafolientriol oxide (BFTO), with a malonyl group at C-3 and acetyl group at C-12 (Fig. 1; Reichardt 1981). Malonic acid is a competitive inhibitor of succinate dehydrogenase (SDH), an enzyme in the tricarboxylic acid cycle in mitochondria that generates cellular energy by ATP production (Lehninger et al. 1993). Therefore, it seemed plausible that PA may be acting through inhibition of SDH, either after hydrolysis by esterases to release malonic acid, or by the malonate group on intact papyriferic acid interacting directly with SDH. The resulting decrease in energy production would constitute a noxious stimulus to the cell. Inhibition of mitochondrial energy production by malonate or other enzyme inhibitors leads progressively to toxicity and cell death (Wallace and Starkov 2000; Fernandez-Gomez et al. 2005). In the gastrointestinal tract, cellular damage leads to the release of serotonin (5-hydroxytryptamine or 5-HT) from enterochromaffin cells, which stimulates 5-HT_3 receptors on vagal afferents, activating neuronal pathways that leads to sensations of nausea, gastrointestinal discomfort, and pain (Spiller 2002; Costedio et al. 2007). Support for this 5-HT mechanism has come from the demonstration that the intake of food containing the *Eucalyptus* antifeedant, jensenone, can be increased in possums after administration of the 5-HT_3 receptor antagonist, ondansetron (Lawler et al. 1998).

In this paper, we report on the metabolism of PA in the rat, and the effect of PA on liver mitochondrial SDH from the rat, ox and rabbit.

Methods and Materials

Chemicals

Papyriferic acid was prepared from the ether extract of the resin of current-year-growth twigs of winter-dormant *B. neoalaskana* saplings (Reichardt 1981). Betulafolientriol oxide (BFTO) was prepared by hydrolysis of PA in alcoholic potassium hydroxide (Reichardt 1981). Other chemicals were purchased commercially and were of analytical reagent grade.

Animals

Rats (Hooded Wistar) and rabbits (New Zealand white) were obtained from the Central Animal House, University of Tasmania. Mitochondria were prepared from livers taken from these animals immediately after sacrifice, and from ox

livers obtained from freshly-killed animals at a local abbatoir. The experiments were approved by the University of Tasmania Animal Experimentation Ethics Committee in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes–2004, 7th Edition.

Dosing

Rats (about 300 g) were treated with 0.1% sodium phenobarbitone in their drinking water for 5 d, then after a washout period of 2 nights, they were gavaged with PA (100 or 500 mg/kg).The 100 mg/kg dose was suspended in mucilage of methyl cellulose, and the 500 mg/kg dose was dissolved in peanut oil (all dose volumes were 0.5 ml). Oil was used as the vehicle for the larger dose to limit possible discomfort to the animals. Food (standard laboratory chow) and water were available ad libitum. Urine and feces were collected separately for 0–24 h and 24–48 h and stored frozen (−20°C) until analyzed.

Analysis of PA and Metabolites

Aliquots of urine were diluted ten-fold with distilled water, buffered to pH 5, and incubated overnight with extract of *Helix pomatia* (β-glucuronidase plus aryl sulfatase; Boehringer Mannheim, Germany) to hydrolyse any conjugates (Boyle et al. 2000). Each 24 h collection of feces was blended with 200 ml water by using a laboratory homogenizer, then a 1 ml aliquot of the fine suspension was extracted with diethyl ether (2×5 ml), and the combined ether extracts taken to dryness at 40°C on a rotary evaporator. The residue was dissolved in 5 ml ethanol. Urine and fecal extracts were filtered (0.45 µ) before analysis by liquid chromatography-mass spectrometry (LC-MS). PA and BFTO were quantitated in fecal extracts by the addition of standards (50–250 µg in ethanol) to 1 ml of the 200 ml homogenates of control 24 h feces taken before dosing.

Metabolites were analyzed initially with a Novapak 150×3.9 mm C18 analytical column and Waters Alliance HPLC coupled to a Finnigan LCQ mass spectrometer using negative electrospray ionization (ESI) conditions as described earlier (McLean et al. 2003). The mobile phase was 85% methanol/ 15% 0.1 M ammonium acetate graded to 100% methanol over a 10 min period, with flow rate 0.8 ml/ min. Metabolites were identified as the products of expected transformations (hydrolysis and hydroxylation of PA) and verified by highly accurate mass measurements using a

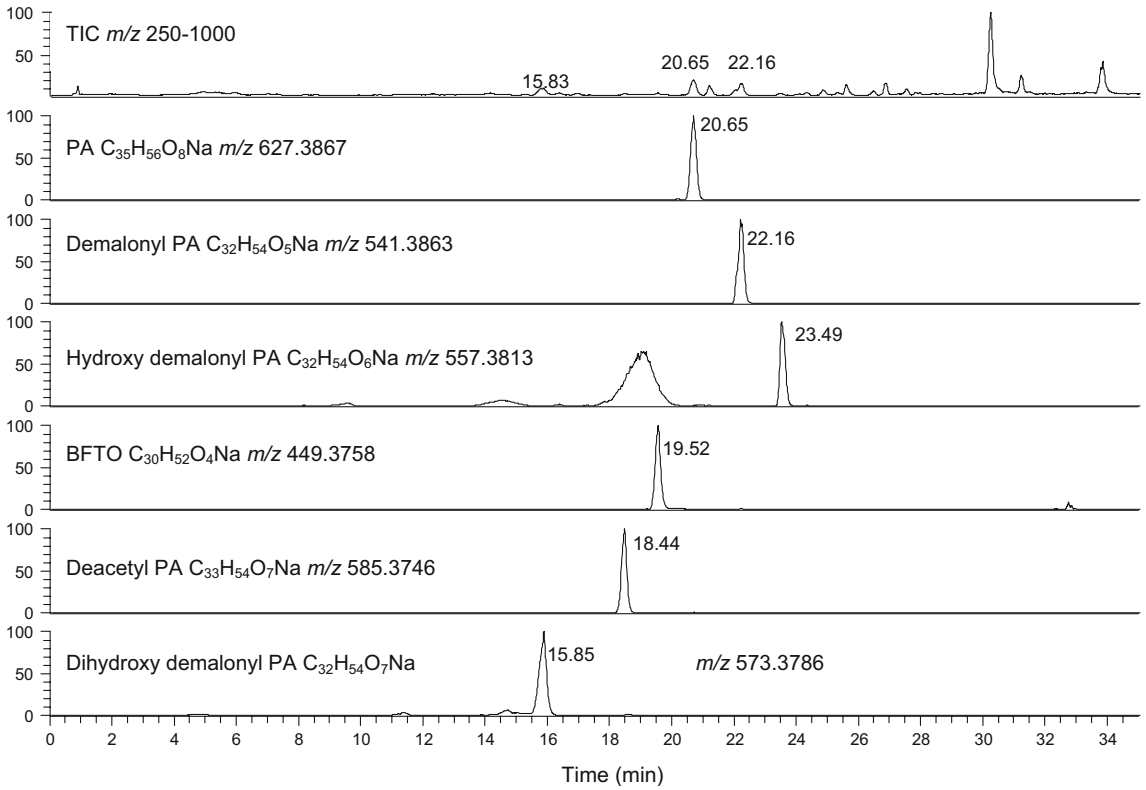


Fig. 2 Chromatograms from LC-MS analysis of rat feces showing TIC and individual mass chromatograms of papyriferic acid and metabolites. The masses are calculated values for the $[M+Na]^+$ ions. The $[M+H]^+$ ion was used in addition for betulafolientriol oxide (*m/z*=477.3957) and deacetyl papyriferic acid (*m/z*=563.3943). Chromatograms are of all measured masses within 6 ppm of the calculated exact masses

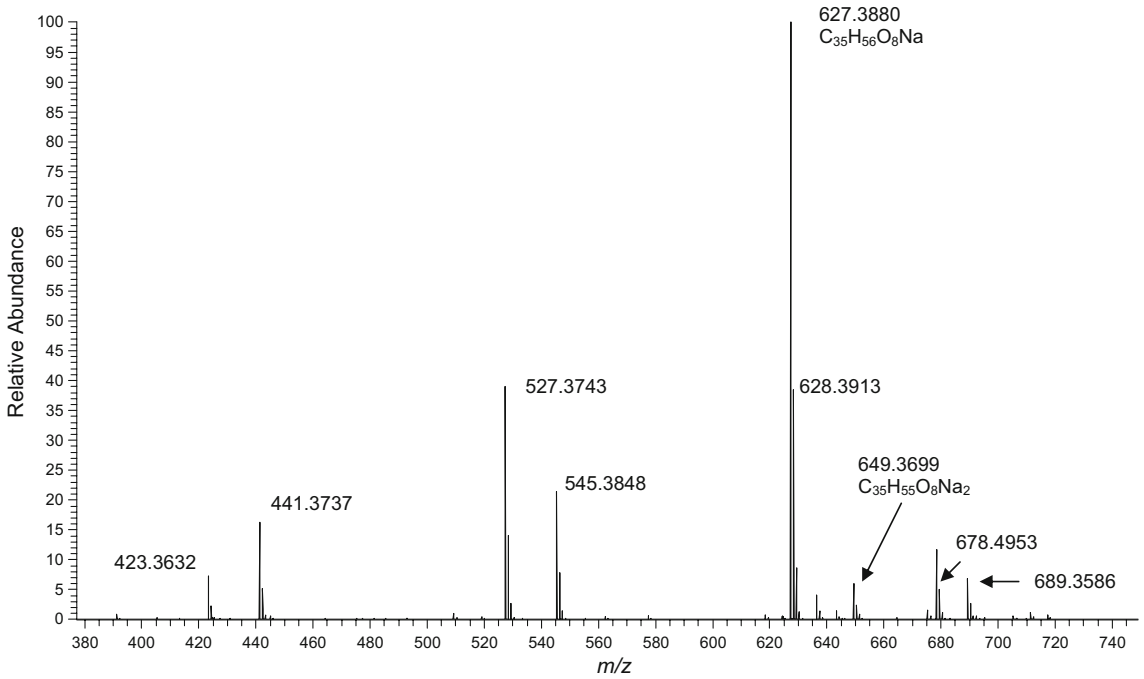


Fig. 3 Mass spectrum of papyriferic acid, showing Na^+-adduct ions measured at *m/z* 627.3880 $[M+Na]^+$ (calculated 627.3867) and *m/z* 649.3699 $[M - H+2Na]^+$ (calculated 649.3686). Note the absence of the protonated molecule $[M+H]^+$ at *m/z* 605

Finnigan Thermo Surveyor HPLC coupled to a Thermo Scientific LTQ Orbitrap mass spectrometer. The column was an Agilent Eclipse Plus C18 (3.5 μ, 2.1×100 mm) and the mobile phase (300 μl/min) 0.1% formic acid in water for 5 min, then graded to 0.1% formic acid in methanol over a 30 min period, then continued for 5 min. MS conditions were: needle voltage 4.5 KV; capillary voltage 20 V; capillary temperature 300ºC; resolution 30,000.

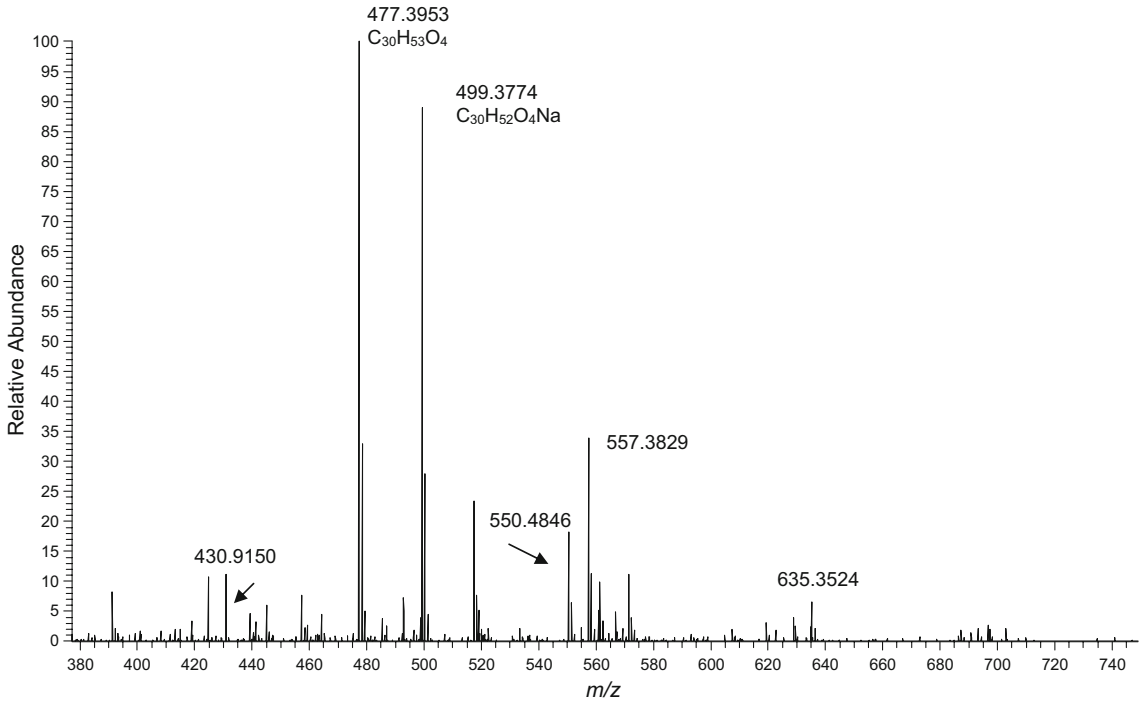


Fig. 4 Mass spectrum of betulafolientriol oxide, showing intense ions measured for both $[M+H]^+$ at *m/z* 477.3953 (calculated 477.3957) and $[M+Na]^+$ at *m/z* 499.3774 (calculated 499.3758)

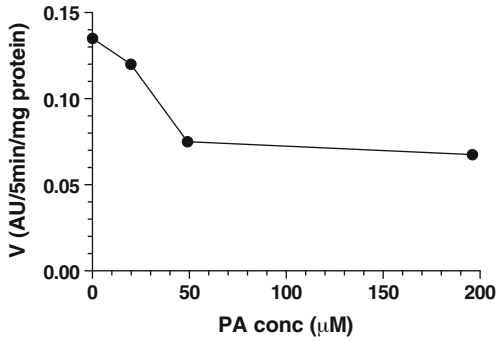


Fig. 5 Inhibition of metabolism of succinate (0.16 mM) by papyriferic acid using ox liver mitochondria

Mitochondrial Incubations

Mitochondrial particles were prepared from fresh liver of ox, rat, and rabbit using the standard method of gentle disruption of tissue and differential centrifugation (Smith 1967), and aliquots were kept frozen at -15ºC until used. The protein content of each was determined by the method of Lowry et al. (1951). Although the term 'mitochondria' is used here, the preparations were not intact but rather particles derived from disrupted mitochondria, which were used as a source of SDH and other mitochondrial enzymes.

Succinic acid was incubated with mitochondria and its oxidation by SDH was measured by the reduction of 2,6-dichlorophenol indophenol (DCPIP), which results in a decreased absorbance at 620 nm (after the method of King (1967) except that phenazine methosulfate was omitted). Reaction rates were measured in a Carey 219 spectrophotometer with cuvette chamber warmed to 25°C. Except for succinic acid, all solutions were kept on ice, and incubation mixtures were prepared at room temperature. PA solutions (20 mM) were prepared each day by dissolution of 6.05 mg in 500 μl ethanol. Incubation mixtures contained NAD^+ 50 μM (250 μl), sucrose Tris buffer 0.25 M pH 7.4 (210 μl), DCPIP 50 μM (1.5 ml), water (to make a final volume of 2.5 ml), PA (if added, 5–10 μl), mitochondria (20 or 40 μl). The spontaneous reaction rate was recorded for 5 min, then the reaction was initiated by the addition of substrate (100 μl). Potassium succinate 1 M was buffered to pH 7.4 and diluted with sucrose-Tris buffer to give the desired final concentration in the incubation mixture (0.1–40 mM). Solutions of malic and malonic acid were prepared similarly. Reaction rates were measured for 5 min, corrected by subtraction of the spontaneous reaction rate before substrate was added, and expressed as AU/5 min/mg protein.

Data Analysis

Plots of reaction velocities and enzyme kinetics were made by using Microsoft Excel and GraphPad Prism software. Plots of reaction velocity (V) vs. substrate concentration (S) were analyzed by non-linear regression to determine the Michaelis-Menten constants, V_{max} and K_m. The mechanism of inhibition was determined by Lineweaver-Burke plots of 1/V vs. 1/S and direct linear plots (DLP) of paired V and S points (Cornish-Bowden 2004). The K_i value for the enzyme-inhibitor-substrate complex (Ki_{EIS}) was determined by a secondary plot of $1/V_{max}$ vs. inhibitor concentration (I). Mean data are given±SE.

Results

Analysis of Standards

LC-MS analysis of PA and BFTO using the LCQ instrument showed single peaks with characteristic ions, as detailed under "Metabolite Identification". The lower limit of detection was well below 1 ng.

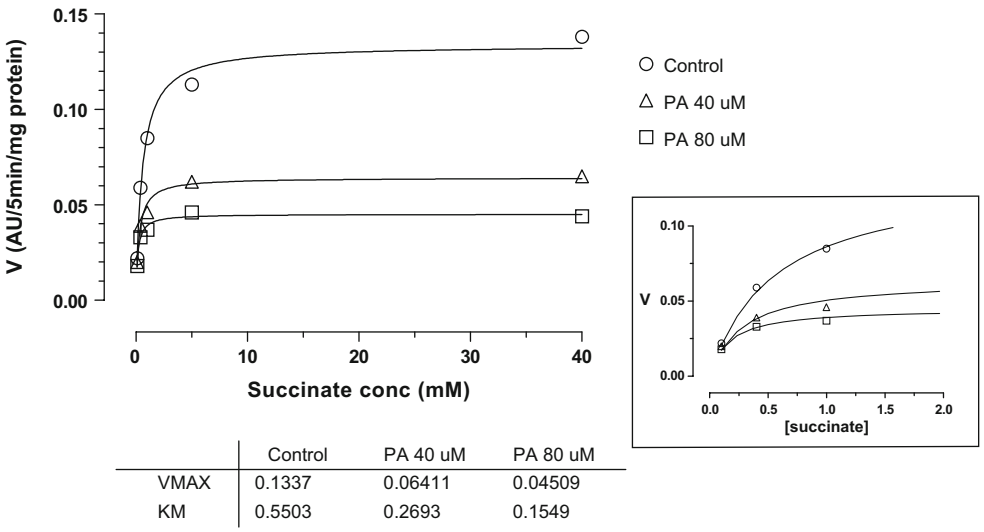
Metabolism of PA in Rats

The rats seemed unaffected after the 100 mg/kg dose, and after the 500 mg/kg dose, they were inactive and lay prone for an hour or two, but later in the day resumed normal activity. By the following day, all rats were eating well and seemed unaffected by the administration of PA. Neither PA nor its metabolites were detected in urine, using the LC-MS methods described below for fecal analyses.

Table 1 Enzyme kinetics of succinate metabolism and its inhibition by papyriferic acid in liver mitochondria from three species

	Succinate alone			Succinate with PA		
Species (N)	V_{max} AU/5 min/mg protein	K_m mM	PA μM	V_{max} AU/5 min/mg protein	K_m mM	K_{iEIS} μM
Ox (4)	0.133±0.013	0.303±0.091	40	0.070±0.008	0.165±0.034	45.27±1.66
Rabbit (3)	0.078±0.006	0.339±0.033	40	0.031±0.002	0.138±0.042	25.67±2.52
Rat (3)	0.064±0.006	0.414±0.086	80	0.020±0.002	0.167±0.110	35.73±2.04

Data given as mean±SE



	Control	PA 40 uM	PA 80 uM
VMAX	0.1337	0.06411	0.04509
KM	0.5503	0.2693	0.1549

Fig. 6 Effect of papyriferic acid (40 and 80 μM) on the metabolism of succinate by ox liver mitochondria. The inset shows detail at low succinate concentrations

Metabolite Identification

PA and its metabolites were observed as $[M+H]^+$ and/or $[M+Na]^+$ ions. Figure 2 shows analysis of a rat fecal extract using the high resolution Orbitrap mass spectrometer, which gives mass measurements to better than 2 ppm. The top panel shows the Total Ion Chromatogram (TIC) monitoring all ions from *m/z* 250–1000 for up to 35 min. From this dataset, mass chromatograms were extracted by selection of the expected exact masses for PA and putative metabolites, and these are shown in the lower panels. These targeted metabolites were based on the expected hydrolysis of ester groups and/or hydroxylation of the triterpene skeleton commonly observed for other xenobiotics (Boyle et al. 2000; Parkinson and Ogilvie 2008). These chromatograms showed single peaks over the narrow mass range (± 6 ppm) selected for display where only one compound was possible. Metabolites readily observed included BFTO, demalonyl PA, deacetyl PA, several hydroxylated demalonyl PAs, and several dihydroxylated demalonyl PAs.

Since all ions had been monitored, it was possible to display the full mass spectrum for each peak, as shown in Figs. 3 and 4. PA did not show an ion at *m/z* 605 ($[M+H]^+$) but did give an intense ion for the sodium adduct $[M+Na]^+$. BFTO, however, showed both ions with approximately equal intensity (Fig. 4) and both were used for the extracted mass chromatogram (Fig. 2) and quantitation.

Tandem MS data acquired on the Finnigan LCQ instrument added further support to the assignments of these metabolites, as the three putative metabolites that retained the acetyl group showed a strong product ion for loss of acetic acid.

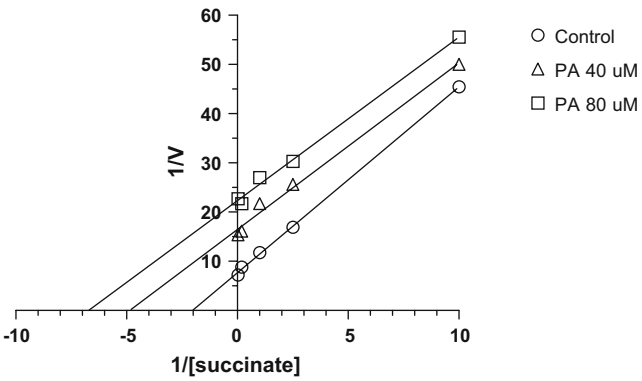


Fig. 7 Lineweaver-Burke plots of data from Fig. 6. The plot is explained in the "Results"

Quantitation of PA and BFTO

Following a dose of 150 mg PA given to two rats (500 mg/kg), the amounts of PA recovered from feces were 52 mg (rat 1) and 5 mg (rat 2) in the 0–24 h collection, and 2 mg and 0 mg, respectively, in the 24–48 h collection. The corresponding recoveries of BFTO were 2 mg and a trace in the first 24 h, and 0.2 mg and 0 mg in the second 24 h. No other metabolites were quantitated, as the standards were not available, and their amounts could not be estimated directly from the ion intensities.

Mitochondrial Incubations

Initial experiments with ox liver mitochondria showed that PA is a potent inhibitor of the mitochondrial oxidation of succinic acid. Figure 5 shows that the velocity of succinate oxidation decreased progressively as the concentration of PA increased from 0 to 200 μM, with marked inhibition at 50 μM.

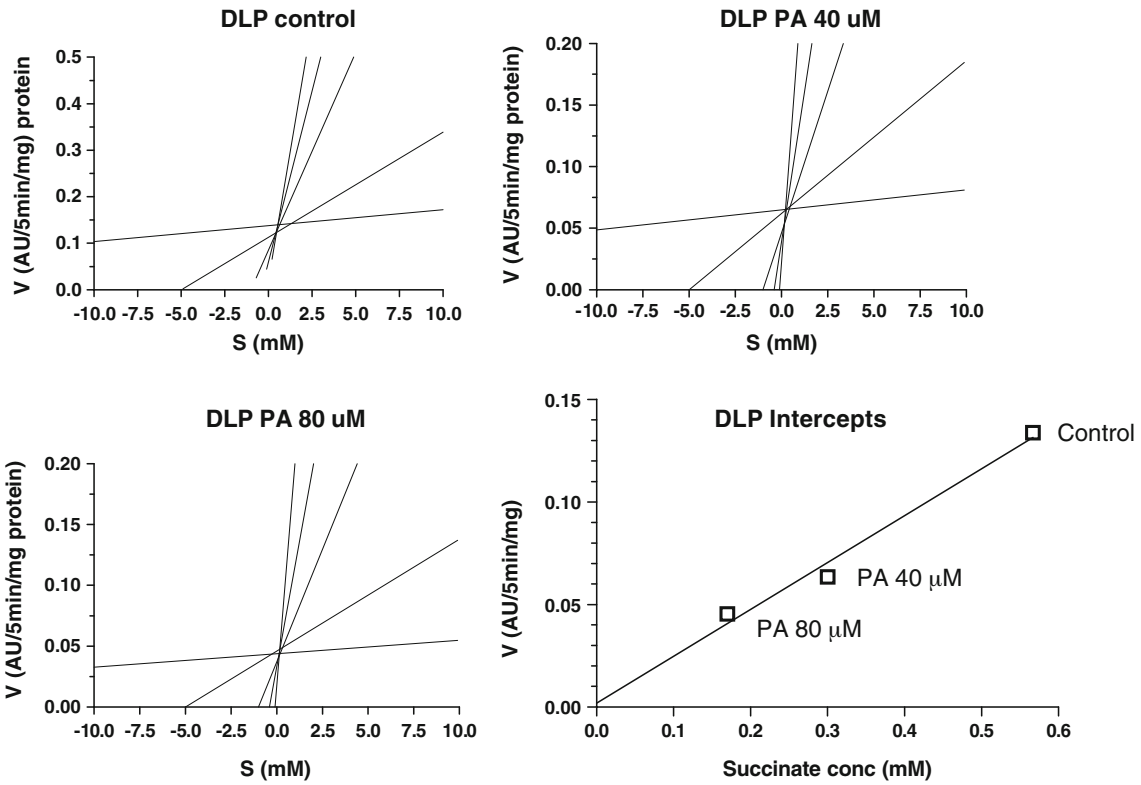


Fig. 8 Direct linear plots of data from Fig. 6 showing intercepts which regressed linearly towards the origin with increasing concentrations of papyriferic acid. The plot is explained in the "Results"

In a series of experiments with liver mitochondria from ox, rabbit, and rat, PA was found to act as an uncompetitive inhibitor of SDH. Enzyme kinetic values for the three species are shown in Table 1. V_{max} is expressed per mg protein to enable comparison between preparations. In all species, both V_{max} and K_m were decreased in the presence of PA. However, rat mitochondria required a higher concentration of PA (80 μM) to show this effect.

Figure 6 shows an example of the V vs. S plot for ox liver mitochondria where PA (40 μM) reduced the maximum reaction rate by more than 50%. At low succinate concentrations (Fig. 6 inset), the inhibition was relatively less complete. The Michaelis-Menten constants calculated from these data are also given in Fig. 6. The Lineweaver-Burke regression lines of these data (Fig. 7)

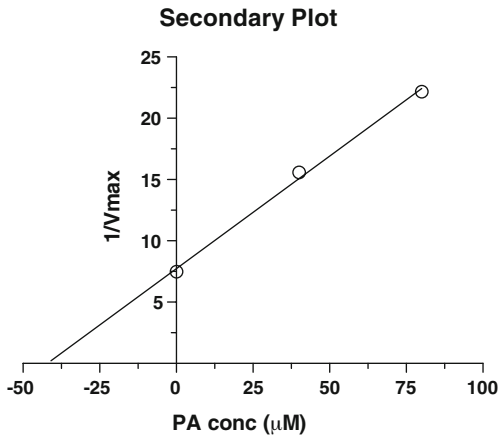
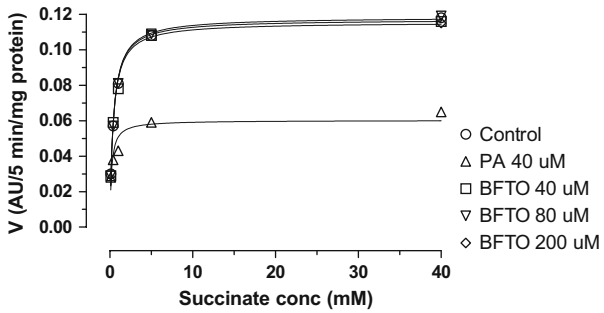


Fig. 9 Secondary plot of data from Fig. 6, showing 1/Vmax vs. concentration of papyriferic acid. The intercept at −42.1 μM gives the K_{iEIS}



	Control	PA 40 uM	BFTO 40 uM	BFTO 80 uM
VMAX	0.1171	0.06019	0.1155	0.1184
KM	0.3966	0.1878	0.3881	0.4117

Fig. 10 Lack of effect of BFTO (betulafolientriol oxide, the hydrolysis product of papyriferic acid) on the metabolism of succinate by ox liver mitochondria. At the highest concentration (200 μM), BFTO was only tested at 40 mM succinate. The inhibitory effect of papyriferic acid is shown as a positive control

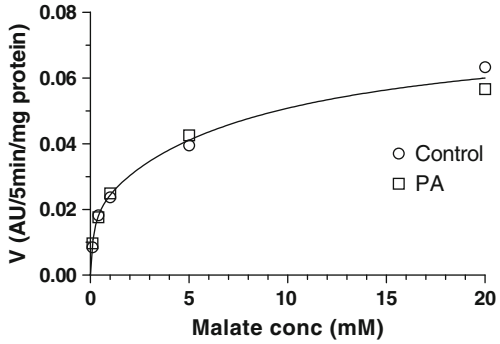


Fig. 11 Lack of effect of papyriferic acid (160 μM) on the metabolism of malate by rabbit liver mitochondria. Similar data were obtained for ox liver (data not shown)

show that PA produced a concentration-dependent parallel shift from the control line, indicating that the mechanism of inhibition was uncompetitive (Cornish-Bowden 2004). This was confirmed by direct linear plots in which paired values of V and S are connected by straight lines (Fig. 8). Each plot (control and two concentrations of PA) produced an intercept of these lines, and a separate plot of these intercept values (Fig. 8: DLP Intercepts) showed a linear regression to the origin with increasing concentration of PA. This is another indication that PA inhibits by an uncompetitive mechanism. A secondary plot of $1/V_{max}$ vs. concentration of PA using the same data (Fig. 9) showed a linear regression with an intercept on the PA concentration axis which gave the value of K_{iEIS} (42.1 μM).

The inhibitory activity was lost when PA was hydrolyzed to BFTO (Fig. 10), indicating that the malonyl group, and possibly the acetyl group, was required for activity. The inhibition was selective for SDH as PA, at an even higher concentration (160 μM), had no effect on malate metabolism (Fig. 11). This shows that malate dehydrogenase was unaffected by PA.

Malonic acid is known to be a competitive inhibitor of SDH, and this was confirmed in mitochondria from each of the species used. Figure 12 shows this for ox liver mitochondria. The Lineweaver Burke plot shows the characteristic pattern for competitive inhibition, with an intercept at $1/V_{max}$. Nonlinear regression analysis of the V vs. S data showed that V_{max} values were similar for control (0.154 AU/5 min/mg protein) and in the presence of 50 μM malonate (0.123 AU/5 min/mg protein) while, as expected for competitive antagonism, the K_m values for succinate differed for control (0.097 mM) and malonate incubations (0.226 mM).

Discussion

The results show that PA is a potent and selective inhibitor of mitochondrial SDH, and that it acts by an uncompetitive mechanism. PA had similar effects in mitochondria from ox, rabbit, and rat, indicating that this is a general effect to be expected in other mammals, including the snowshoe hare and other animals that browse on twigs of birch that produce PA (e.g., the juveniles of *B. neoalaskana* and *B. pendula*, Reichardt et al. 1984; Rousi et al. 1991; Tahvanainen et al. 1991; Julkunen-Tiitto et al. 1996; Pusenius et al. 2002; Bryant et al. 2009). This study does not prove that inhibition of SDH underlies the anti-feedant effect of PA, but this mechanism seems likely. As discussed in the "Introduction", noxious stimuli, such as those produced by inhibition of mitochondial energy supply, can release serotonin in the gut leading to sensations such as nausea, which would suppress feeding (Spiller 2002; Costedio et al. 2007). In vivo, mitochondria are protected by membrane barriers of the host cell and the organelle itself, but PA is a lipophilic substance and would be expected to readily permeate biological membranes.

Although the lack of urinary metabolites indicates that PA is poorly absorbed into the bloodstream from the gut, its potency in the in vitro experiments suggests that only a small amount would need to enter the gut cells to inhibit mitochondrial activity and elicit an antifeedant response.

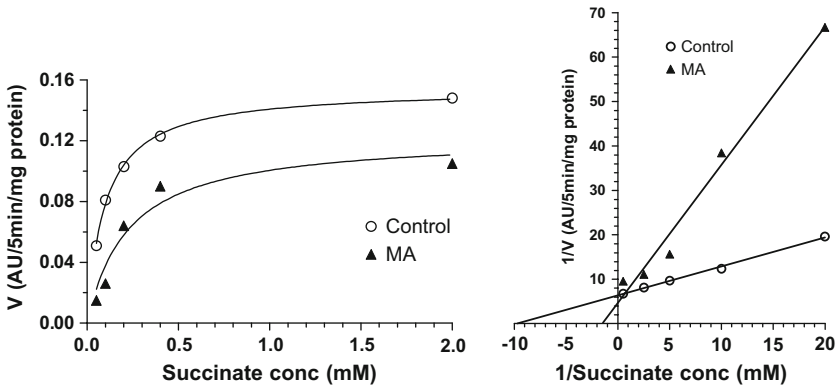


Fig. 12 Effect of malonic acid (MA, 50 μM) on the metabolism of succinate by ox liver mitochondria

Poor absorption may also explain why, despite its potency in vitro as an enzyme inhibitor, hares can ingest relatively large doses of PA before ceasing feeding (Reichardt et al. 1984; Bryant et al. 1994, 2009).

The intestinal epithelium possesses the P-glycoprotein efflux transporters that are expressed at the apical surface of enterocytes (Kullak-Ublick and Becker 2003) and can regulate the systemic absorption of plant secondary metabolites (Sorensen and Dearing 2006). However, the cycle of diffusion into the enterocyte and transport back into the lumen of the intestine effectively prolongs the cellular residence time and increases the extent of interaction with cellular enzymes (Johnson et al. 2001). This would promote both the inhibition of SDH by PA, and the possible metabolism of PA by cellular enzymes.

PA also is a steroid and is, therefore, a possible substrate for the hepatic organic anion transporter, which takes bile acids, endogenous steroid hormones, and plant steroids (e.g., digoxin) from the blood into bile (Kullak-Ublick and Becker 2003). This would also act to decrease the systemic absorption of PA while exposing it to the xenobiotic metabolizing enzymes of the liver. A full pharmacokinetic study is required to resolve these questions.

Although the metabolic study found that in vivo at least some of the PA loses its malonyl group by hydrolysis, it seems likely that PA acts as the intact molecule rather than by releasing malonic acid. First, as shown in this study, PA and malonate inhibit SDH by two different mechanisms (uncompetitive and competitive, respectively). Second, the inhibition of ox liver SDH by 50 μM malonate was less than that by 40 μM PA, and even if all of the PA were hydrolyzed (an unlikely event given the chemical stability of PA to hydrolysis and absence of appropriate enzymes in mitochondria), the concentration of malonate could not exceed 40 μM.

The uncompetitive mechanism of action, in which the inhibitor binds to the enzyme-substrate complex, has the consequence that inhibition is relatively greater at high substrate concentrations, and that even at high concentrations of inhibitor the reaction is not completely inhibited (as shown in Fig. 5). Similarly, at low succinate concentrations, inhibition is not complete (Fig. 6, inset). This suggests that, unlike malonate, which causes dose-dependent neurotoxicity (Fernandez-Gomez et al. 2005), PA will not produce irreversible toxic effects. This is supported by the observations in rats given high oral doses of PA, which showed temporary discomfort followed by a full recovery the next day.

The original hypothesis that PA, like malonate, interacts with the succinate binding site on SDH must be discarded, since the mechanistic evidence indicates that PA does not bind in place of the substrate but to the enzyme-substrate complex. Recent evidence suggests where this site may be. α-Tocopherol succinate (TOS) is a selective inducer of apoptosis in cancer cells, and now has been shown to act through the inhibition of SDH activity of complex II by interacting with the ubiquinone binding sites (Dong et al. 2008). Like PA, TOS has a lipophilic (C_{29}) region, which is esterified with a dicarboxylic acid, and in both cases activity is lost after hydrolysis. Further study will be required to determine whether PA acts through binding to the ubiquinone sites, and this could be commenced by using molecular modeling since the crystal structure of complex II has been described (Sun et al. 2005).

Although data are presented on three species, this study was not designed to compare the effects of PA in different species, which requires more rigorous preparation and characterization of mitochondria (Hulbert et al. 2006). There can be dramatic differences among species in the susceptibility of their mitochondrial enzymes to inhibition (Krueger et al. 1995). If these differences also occur as variations among geographically separated congeners or conspecifics, then this would be an important topic for future study, for it would provide a biochemical test of Thompson's (2005) geographic mosaic of selection hypothesis.

A geographic mosaic of birch-hare coevolution may exist across the vast boreal forest and north temperate forest of North America (Bryant et al. 2009). This suggests that a transcontinental comparison of the PA tolerance of snowshoe hares from regions home to birches with PA concentrations ranging from high to low is warranted, especially given the unprecedented spatial scale at which these terrestrial mosaics could operate relative to other previously documented consumer-resource mosaics (e.g., Thompson 2005; Thompson and Fernandez 2006; Toju and Sota 2006; Siepielski and Benkman 2007; Toju 2008). The comparison would be a transcontinental test of Thompson's (2005) geographic mosaic of coevolution hypothesis.

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which is a very different from the emissions of healthy plants (Kännaste et al. 2008). In the present study, we compared the volatile blends released by a Norway spruce clone infested either by *Nalepella* sp. Boczek var. *Picea abietis* (Acarina, Ereophyidae) (Phytoptidae) (Löyttyniemi 1973) or by *Oligonychus ununguis* Jacobi (Acarina, Tetranychidae) (Jeppson et al. 1975). For a control, volatiles from an uninfested spruce clone also were analyzed. Both of the mite species can be serious pests of conifers in nurseries (Ehnström et al. 1974; Lehman 1982) and, due to their rapid development from larval to adult stages, five or more generations of *Nalepella.* sp. (Löyttyniemi 1973; Lehman 1982) can develop per year.

Pine weevils are attracted to weakened trees, and as the mite-infested, small plants must be regarded as highly stressed, we studied whether or not the main odors emitted by heavily infested plants were attractive to the weevils. The major compounds emitted by mite-induced spruce were tested separately and in combination with host odors in two multi-choice laboratory bioassays.

Methods and Materials

Biological Material Potted *Picea abies* plants of clone 1321, 4–5 yr-old and ca. 30 cm tall, originating from the clone archives maintained by Skogforsk (the Forestry Research Institute of Sweden), and two species of mites, identified as *Nalepella* sp. and *Oligonychus ununguis*, were used in this study. Mites of each species were fed separately for a year on the needles of a set of 8 plants. Plants were provided with artificial daylight (18 h light/6 h dark cycles, 20°C) and watered weekly. At the end of this period, both mite species completely infested the plants (approx. density: 1–5 mites /cm^2). A set of control plants was kept mite-free throughout this period.

Pine weevils (*Hylobius abietis*) of both sexes were collected during spring migration at sawmills in central Sweden. After collection, the weevils were stored in darkness at 10ºC and provided with fresh conifer bark as food. These conditions interrupted their reproductive development. They then were transferred to a chamber where they were kept for at least 10 d at 18 h light/6 h dark cycles, and 20°C prior to the bioassays. Oviposition generally commenced about a week after the transfer to the chamber. The weevils had continuous access to conifer bark as food until 24 h before use in the bioassay.

Collection of Spruce Volatiles The volatile compounds released by 4 plants infested by a mite species and 4 controls were collected by solid-phase microextraction (SPME) as follows. Each plant was enclosed in a plastic bag (polyethylene terephthalate, Toppits Melitta, 35× 43 cm). A fused silica fiber coated with a 65 μm film of polydimethylsiloxane/divinylbenzene (Supelco, Bellefonte, PA, USA) was inserted into the bag to collect the volatiles emitted from the living plant. Volatiles were collected over a 24 h period for GC-MS analysis and a 5 h period for 2D-GC-analysis (see below).

Separation and Identification of Volatiles The chemical composition of the volatiles in each headspace was analyzed using a Varian 3400 gas chromatograph (GC) connected to a Finnigan SSQ 7000 mass spectrometer (MS) operated in electron ionization, full-scan mode (ionization energy, 70 eV; injector temperature, 215°C; ion source temperature, 150°C; 30 s splitless injections; mass range 33–400 m/z). The GC was equipped with a CB1 column (Supelco, 30 m, 0.25 mm id and 0.25 μm film thickness). Analytes were eluted using the following temperature program: 40°C held for 1 min, followed by a 4°C/min gradient to 190°C, held for 0.01 min, then a 20°C/min gradient to 220°C, held for 10 min; helium (pressure 10 psi) was used as the carrier gas. Spectra in the NIST (National Institute of Standards and Technology) and MassFinder 3.06 reference libraries were used in conjunction with published retention indexes (RIs) and mass spectra of available reference samples for identifying the spruce volatiles (Davies 1990; Ruther 2000).

In addition, the enantiomeric compositions of the chiral terpenes that strongly affect the pine weevil's behavior were analyzed by 2D-GC (Borg-Karlson et al. 1993). In the first GC, a DB-wax column (J&W Scientific™, 30 m, 0.25 mm id, 0.25 μm film thickness) was used with an injector temperature of 215°C (30 s splitless injections), and the following temperature program: 40°C held for 2 min followed by a 4°C/min increase to 130°C, held for 12 min, a further 4°C/min rise to 150°C, held for 0.01 min, then a final 10°C/min gradient to 220°C, held for 10 min. In the second GC, an HP Chiral-20 β 19091G-B233 (J&W, 30 m×0.25 mm×0.25 μm, 20%) was used to separate the chiral constituents using a temperature program of 55°C for 11 min, followed by a 1°C/min increase to 75°C, held for 65 min, and finally a 1°C/min rise from 75°C to 90°C, held for 60 min. Helium was used as the carrier gas at a pressure of 34 psi for the first and 22 psi for the second GC. Both GCs were equipped with a flame ionization detector (FID), and the temperature of the FIDs during the analyses was 250°C.

Chemicals Standards that were not already available in our laboratories were purchased, at the highest purity offered (>98%) from Lancaster Synthesis or Sigma-Aldrich.

Preparation of Dispensers Capped polyethylene tubes (1.5 ml, 1 mm thick walls) were used for dispensing (-)-

linalool and (*E*)-β-farnesene, while 1 ml tubes with 2 mm walls were loaded with MeSA for the bioassays with the pine weevil. Due to the poor emission of (*E*)-β-farnesene, a metal needle (0.5 mm diam.) was used to puncture 10 holes in the walls of each dispenser. In each tube, 10 µl of a single compound were added.

Emissions from Loaded Dispensers The emission rates from the dispensers loaded with (-)-linalool, (*E*)-β-farnesene, and MeSA in dynamic conditions were checked before use in the bioassays by hanging one dispenser containing each of the three substances in a 1.8 liter glass jar, and pumping air into the jar using a 224-PCXR7 SKC universal pump (SKC) at room temperature through a Porapak Q filter with a flow rate of 450 ml/min. After 2 d of stabilization, a fused silica fiber with a 65 µm coating of polydimethylsiloxane/divinylbenzene (Supelco, Bellefonte, PA, USA) was inserted into the jar to trap the volatiles released during 1 h.

Laboratory Bioassay with H. abietis The behavioral response of *H. abietis* to three mite-induced compounds (linalool, (*E*)-β-farnesene, and MeSA) was examined in two experiments in a multi-choice arena with 24 pitfall traps. The first experimental set-up included 18 traps that contained one dispenser each (six for each compound) and six control traps with no odor dispenser. The second experiment differed from the first in that a freshly cut piece of Norway spruce twig (0.5 cm diam., length 2 cm) was suspended in each of the 24 traps. Thus, the second experiment measured the response of *H. abietis* to each of the three compounds in combination with a blend of attractive host volatiles, mainly monoterpenes. Each of the two experiments was run four times, twice with 50 males and twice with 50 females. Each run lasted 24 h, after which the numbers of weevils that had entered each of the 24 traps were counted.

The arena was made of black Perspex and covered an area of 100×70 cm, with 14 cm high sides painted with Fluon RAD 1090 (AG Fluoropolymers, Thornton, Lancashire, UK) to prevent weevils from climbing up. We uniformly distributed 24 conical plastic jars into holes (65 mm) on the bottom of the arena. Each jar was capped with a plastic lid under which the dispenser (and the piece of spruce in experiment 2) was suspended, out of reach of the weevils. The jar extended 30 mm above the arena floor. Eight holes (14 mm diam.) with their lower edge 5 mm above the floor of the arena were equally spaced around each jar. Thus, the weevils in the arena had to actively climb up 5 mm to enter a hole and fall into the trap. The beetles entering a trap fell through a funnel attached to the open bottom of the jar and into a container 40 cm below the arena. According to results of separate tests, these captured weevils did not affect the likelihood of additional weevils entering the traps.

In each of the two bioassays with *H. abietis,* the significance of differences in the data obtained for the males and females was tested by heterogeneity *chi-square* analysis, and it was found that these data could be validly pooled. The *chi-square* goodness of fit of the pooled data was then calculated with the null hypothesis of equal numbers of weevils entering the differently baited traps.

Results

Emissions from Healthy and Infested Spruces Large differences in the composition of emitted volatiles were found between the healthy and heavily mite-infested spruces (Table 1, Fig. 1). Limonene was the dominant monoterpene in the emissions of healthy spruces, especially the (-)-enantiomer, which accounted for 80% of the limonene content (Table 1). Feeding by both species of mites induced the release of several compounds, not detected in the emissions from the uninfested controls, including the terpenes (*Z*)-β-ocimene, 2,6-dimethyl-2,6-octadiene, 4,8-dimethylnona-1,3,7-triene (DMNT), *cis*- and *trans*-linalooloxides (furane type), neral, geraniol, geranial, *trans*-α- and *trans*-β-bergamotene, (*Z*)-β-and (*Z*,*E*)-α-farnesene (Table 1). MeSA and (*E*,*E*)-α-farnesene were the main compounds in the bouquet of *Oligonychus ununguis*-infested spruces, while (*E*)-β- and (*E*,*E*)-α-farnesene dominated the emissions of *Nalepella* sp.-attacked spruce plants (Table 1, Fig. 1). In addition, both mite infested spruce specimens released linalool with similar enantiomeric composition; the (-)-enantiomer accounting for ca. 95% of the total linalool released.

Among many other compounds, spruces attacked by the mites released several green leaf volatiles (GLV:s), e.g., (3*Z*)-hexenol, (3*Z*)-hexenyl acetate and hexyl acetate, and various aromatic compounds, e.g., benzaldehyde, benzylalcohol, benzyl acetate, 1,3-dimethoxybenzene, benzoic acid, estragole, and vanillin (Table 1).

Comparison of the Emissions from Spruces Infested by the Two Mite Species Only grafts infested with mites of *Nalepella* sp. emitted: (3*Z*)-hexenol, (3*Z*)-hexenyl acetate, and hexyl acetate; the oxygenated monoterpenes *cis*-linalool (furanoid) and linalyl acetate; and the sesquiterpenes (*Z*)-β-farnesene, *trans*-α-bergamotene, and *trans*-β-bergamotene. Neral was emitted only from *O. ununguis*-infested spruces (Table 1). The spruces were infested for a year before the experiment, and the infestation was regarded to be in a steady state, with a fairly even density of mites. Nevertheless, there were substantial between-plant variations in the

Table 1 Volatiles released by the healthy and infested spruces of clone 1321

No	Compound	Ret. time, min	RI, calc.	Healthy spruces, *N*=4		Infested spruces			
						N. sp., *N*=4		*O. ununguis*, *N*=4	
1	3-Methyl-2-butenal	4.57	805	–	–	t	4/4	t	3/4
2	(3*Z*)-hexenol	6.42	861	–	–	t	4/4	–	–
3	Unknown, B 41, 69; MW 98	8.00	896	–	–	t	4/4	t	3/4
4	Benzaldehyde	9.13	930	–	–	t	4/4	t	4/4
5	α-Pinene	9.20	933	+	4/4	–	–	–	–
6	Camphene	9.61	945	t	4/4	–	–	t	2/4
7	*o*-Cymene	10.28	964	t	3/4	–	–	–	–
8	Sabinene	10.45	968	t	3/4	–	–	–	–
9	6-Methyl-5-heptene-2-one	10.48	968	+	1/4	t	4/4	t	4/4
10	β-Pinene	10.54	968	t	3/4	–	–	–	–
11	Myrcene	11.15	986	+	4/4	t	4/4	t	3/4
12	(3*Z*)-Hexenyl acetate	11.32	990	–	–	t	4/4	–	–
13	Hexyl acetate	11.62	997	–	–	t	4/4	–	–
14	2-Ethenyl-1,1-dimethyl-3-methy-lenecyclohexane	11.71	999	–	–	t	4/4	–	–
15	3-Carene	11.84	1004	+	4/4	t	4/4	–	–
16	Benzylalcohol	11.92	1006	–	–	t	4/4	t	4/4
17	*m*-Cymene	12.04	1009	t	1/4	–	–	–	–
18	*p*-Cymene	12.16	1013	+	4/4	t	4/4	t	3/4
19	β-Phellandrene	12.40	1020	+	4/4	t	3/4	–	–
20/21	(-/+)-Limonene	12.47	1022	++	4/4	t	4/4	t	4/4
22	(*Z*)-β-Ocimene	12.74	1030	–	–	t	4/4	t	2/4
23	2,6-Dimethyl-2,6-octadiene	12.91	1034	–	–	t	4/4	+	4/4
24	(*E*)-β-Ocimene	13.21	1043	t	4/4	t	4/4	t	2/4
25	γ-Terpinene	13.55	1052	t	4/4	–	–	–	–
26	*trans*-Linalooloxide (furanoid)	13.79	1058	–	–	t	4/4	t	1/4
27	1-Octanol	13.93	1062	+	4/4	t	2/4	t	4/4
28	*o*-Cymenene	14.20	1069	t	3/4	–	–	–	–
29	Methyl benzoate	14.30	1072	+	4/4	t	4/4	t	4/4
30	*cis*-Linalooloxide (furanoid)	14.36	1073	–	–	t	3/4	–	–
31	*p*-Cymenene	14.44	1075	t	3/4	–	–	–	–
32	Terpinolene	14.65	1080	+	4/4	–	–	–	–
33/34	(−/+)-Linalool	14.84	1085	–	–	+	4/4	t	4/4
35	Aromatic compound*, B 43, 77, 91, 93	15.23	1094	–	–	t	4/4	t	2/4
36	4-Acetyl-1-methylcyclohexene	15.52	1101	–	–	t	4/4	t	2/4
37	DMNT	15.66	1106	–	–	t	4/4	t	4/4
38	Unknown, B 95, 43, 110; MW 152	16.08	1118	t	1/4	–	–	–	–
39	Camphor	16.12	1119	t	4/4	–	–	–	–
40	Aromatic compound, B 134, 133, 119, 105, 91; MW 134	16.52	1124	–	–	t	4/4	t	4/4
41	Camphene hydrate	16.54	1131	t	4/4	–	–	–	–
42	Benzyl acetate	16.66	1134	–	–	t	4/4	t	1/2
43	1,3-Dimethoxy-benzene	16.72	1136	–	–	t	2/4	–	–
44	Borneol	17.20	1149	+	3/4	–	–	t	4/4
45	Benzoic acid	17.53	1158	–	–	t	4/4	+	4/4
46	Methyl salicylate	17.95	1169	+	3/4	+	4/4	++	4/4
47	α-Terpineol	18.10	1173	t	3/4	t	1/4	–	–
48	Estragole	18.12	1173	–	–	t	2/4	t	2/4
49	Neral	19.62	1213	–	–	–	–	t	4/4

Table 1 (continued)

No	Compound	Ret. time, min	RI, calc.	Healthy spruces, *N*=4		Infested spruces			
						N. sp., *N*=4		*O. ununguis*, *N*=4	
50	Methylthymylether	19.74	1217	t	3/4	t	3/4	–	–
51	Geraniol	20.42	1237	–	–	t	4/4	t	3/4
52	Linalyl acetate	20.60	1242	–	–	t	4/4	–	–
53	Geranial	20.64	1242	–	–	t	4/4	t	4/4
54	Unknown, B 69, 41	21.06	1255	–	–	t	4/4	t	4/4
55	Bornyl acetate	21.57	1270	+	4/4	t	4/4	t	4/4
56	γ-Nonanolide	23.34	1317	t	4/4	t	4/4	t	4/4
57	α-Terpinyl acetate	23.70	1330	+	4/4	t	3/4	t	3/4
58	α-Longipinene	24.28	1348	t	4/4	–	–	–	–
59	MT-O, B 93, 55, 41, 85; MW 178	24.36	1340	–	–	+	4/4	t	1/4
60	Vanillin	24.39	1351	–	–	t	4/4	t	4/4
61	Cyclosativene	24.98	1369	+	4/4	–	–	–	–
62	α-Copaene	25.15	1373	t	3/4	–	–	–	–
63	SqT, B 119, 93, MW 204	25.35	1379	t	1/4	–	–	–	–
64	β-Bourbonene	25.37	1380	t	1/4	–	–	–	–
65	Sativene	25.57	1386	t	3/4	–	–	–	–
66	MT-O, B 119, 93, 41, 79, 85; MW 188	25.75	1390	–	–	t	4/4	t	3/4
67	Longifolene	26.02	1398	+	4/4	–	–	–	–
68	7-Epi-α-cedrene	26.25	1404	t	3/4	–	–	–	–
69	MT-O, B 119, 93, 41, 80, 85, 55; MW 178	26.29	1406	–	–	t	4/4	+	4/4
70	(*E*)-β-Caryophyllene	26.47	1412	+	4/4	–	–	–	–
71	(*Z*)-β-Farnesene	26.75	1421	–	–	t	4/4	–	–
72	Geranylacetone	26.94	1427	t	4/4	–	–	+	4/4
73	*trans*-α-Bergamotene	26.98	1429	–	–	t	4/4	–	–
74	Sesquisabinene A	27.11	1433	+	4/4	t	4/4	t	1/4
75	SqT, B 119, 94, MW 204	27.39	1442	t	4/4	–	–	–	–
76	α-Humulene	27.51	1446	+	4/4	–	–	–	–
77	(*E*)-β-Farnesene*	27.67	1450	t	4/4	+++	4/4	+	4/4
78	1-Dodecanol	28.02	1462	t	4/4	–	–	–	–
79	SqT-O, B 93, 41, 55, 79, 119; MW 220	28.23	1468	–	–	t	4/4	t	1/4
80	SqT, B 119, 132, 105, 93; MW 204	28.29	1470	t	4/4	–	–	–	–
81	Germacrene D	28.50	1476	–	–	t	1/4	–	–
82	*trans*-β-Bergamotene	28.48	1476	–	–	t	4/4	–	–
83	(*Z*,*E*)-α-Farnesene	28.70	1483	–	–	+	4/4	+	4/4
84	(*E*,*E*)-α-Farnesene	29.20	1497	+	4/4	++	4/4	+++	4/4
85	β-Bisabolene	29.27	1499	t	1/4	–	–	–	–
86	δ-Cadinene	29.65	1512	t	4/4	–	–	–	–
87	(*E*)-α-Bisabolene	30.02	1531	+	4/4	+	4/4	+	4/4
88	SqT, B 41, 69, 93, 135; MW 218	31.30	1567	–	–	t	4/4	t	1/4
89	Benzophenone	31.83	1584	+	4/4	–	–	t	3/4
90	SqT, B 119, 41, 134; MW 218	33.60	1645	–	–	t	4/4	+	4/4
91	An Heptadecadiene*	34.14	1664	–	–	+	4/4	t	3/4
92	Unknown B 79, 108, 80, 41, 67	34.24	1667	+	4/4	–	–	–	–
93	An Heptadecene*	34.45	1674	–	–	t	4/4	t	3/4

* - T-test, $P<0.05$; t=<1%;+ =1–10%; ++ =10–30%; +++ = >30% of the sum of peak areas of volatiles; x/4=compound detected x times of 4 analyses.

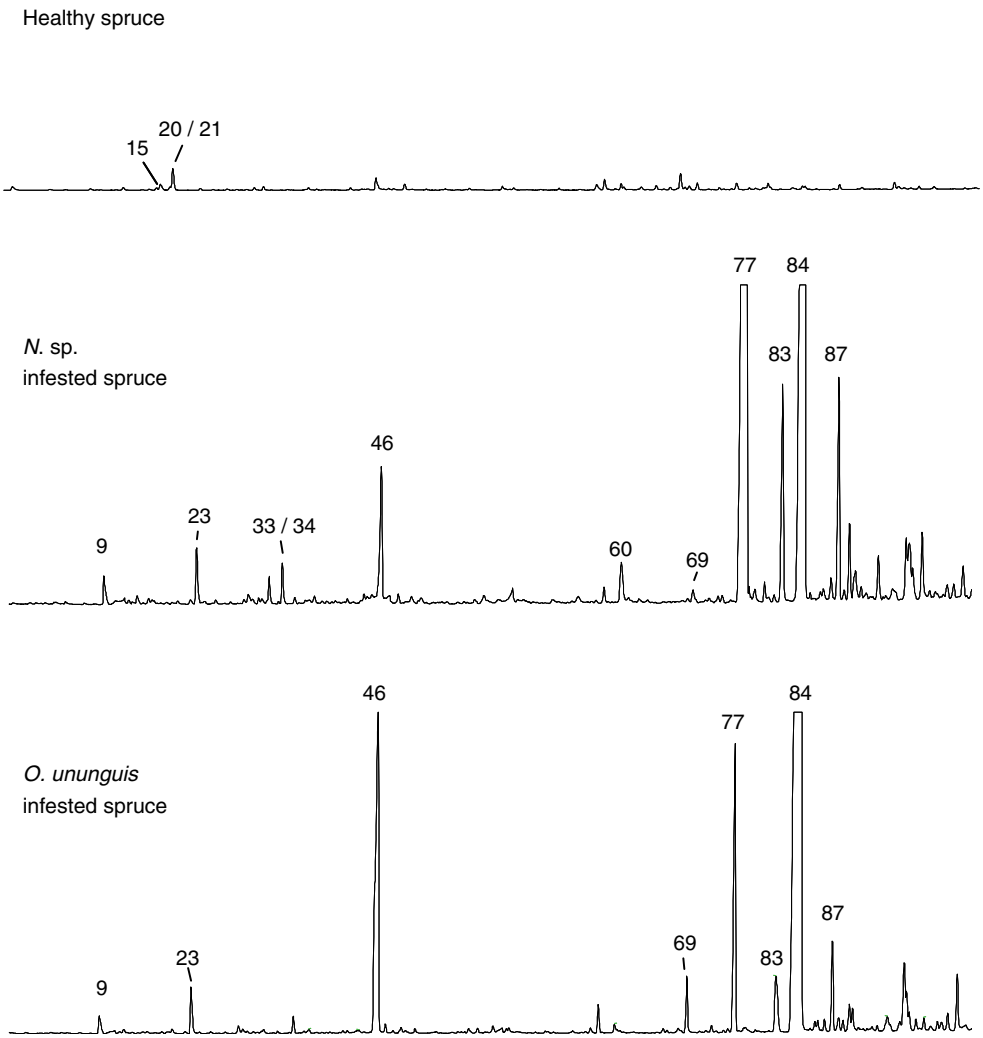

Fig. 1 Representative chromatograms of 4–5 yr-old spruces of clone 1321. Identified compounds — 9, 6-methyl-5-heptene-2-one; 15, 3-carene; 20/21, limonene (80% of (-)-enantiomer); 23, 2,6-dimethyl-2,6-octadiene; 33/34, linalool (95% of (-)-enantiomer); 46, methyl salicylate (MeSA); 60, vanillin; 69, unidentified oxygenated monoterpene (MT-O) B 119, 93, 41, 80, 85, 55; MW 178; 77, (*E*)-β-farnesene; 83, (*Z*,*E*)-α-farnesene; 84, (*E*,*E*)-α-farnesene; 87, (*E*)-α-bisabolene

relative amounts of volatiles induced by the mites. Despite this variation, certain compounds could be regarded as being mite-specific (Table 1, Fig. 1).

The profiles of the emissions of aromatic compounds from infested plants seemed to be related to the species of mite. Vanillin was present in small amounts in the emissions from both types of infested spruces. Feeding by *O. ununguis* and *Nalepella* sp. mites triggered increases in the emissions of MeSA compared to healthy spruce. However, MeSA was present in larger amounts in the emissions of spruce attacked by *Naplepella.* sp.

Release Rate of Volatiles by Dispensers In dynamic air conditions, stabilization time was required to allow the rate of release of compounds from the dispensers to reach a steady state (Fig. 2). After this time, according to the calibration, the dispensers emitted on average 20 ng of the test compound per h. These dispensers were used in the laboratory bioassays to assess the effects (attraction/repellence) of the compounds on the behavior of pine weevils. Under similar conditions, we estimated that the *Nalepella* sp.-infested spruces released approximately 1, 11, and 8 ng per h (-)-linalool, (*E*)-β-farnesene, and MeSA, respectively.

Results of Laboratory Bioassays with H. abietis The *Chi-square* analysis of the pooled data for each of the two experiments showed that the distribution of captured weevils differed significantly from an even distribution between the differently baited traps (*Chi-square*=23.9, *df*=3, $P<0.001$ for experiment 1 and *Chi-square*=19.7, *df*=3, $P<0.001$ for experiment 2) (Fig. 3). In the first experiment, when the three substances were presented without the addition of host volatiles, (*E*)-β-farnesene attracted both male and female *H. abietis*. A similar tendency was found for (-)-linalool, but MeSA did not appear to elicit any behavioral response. When the compounds were presented together with spruce odor, both sexes were attracted less strongly to the traps with both MeSA and (-)-linalool than they were to the control traps with host plant odor alone. (*E*)-β-farnesene did not affect the attraction of *H. abietis* to host odor.

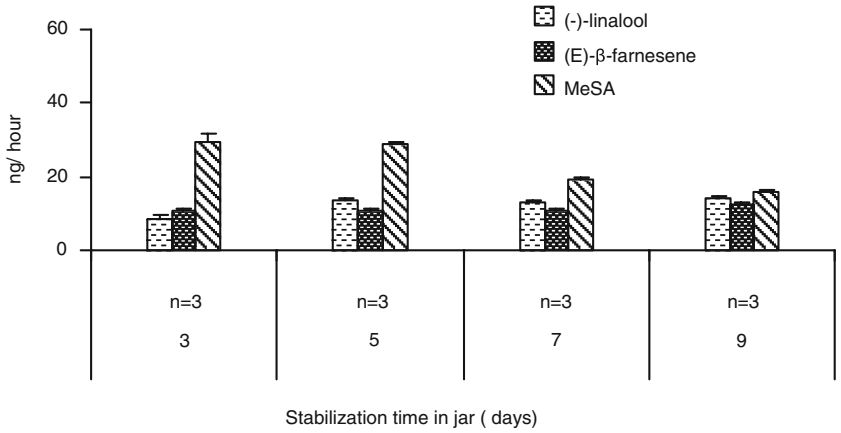

Fig. 2 Release rates (mean±SD) of (-)-linalool, (*E*)-β-farnesene, and MeSA from dispensers in flowing air. The mean emission rates of the compounds were obtained from three measurements. Capped polyethylene tubes, with 1 mm thick walls were used with (-)-linalool and (*E*)-β-farnesene, while tubes with 2 mm thick walls were loaded with methyl salicylate. Release of (*E*)-β-farnesene was increased by perforating the walls of the tubes containing it

Discussion

We have previously shown that clones of small conifer plants infested by mites release many kinds of compounds, especially terpenes (Kännaste et al. 2008). Arimura et al. (2000a, b) showed that DMNT and (*E*)-β-ocimene released by bean plants attacked by the spider mite *Tetranychus urticae* activate genes involved in the induced defense reaction of intact plants. Moreover, green leaf volatiles, (3*Z*)-hexenol and (3*Z*)-hexenyl acetate, which are also released during ozone damage, have been claimed to activate the defense genes of neighboring intact plants (Arimura et al. 2001; Heiden et al. 2003). In addition, Shulaev et al. (1997) postulated that methyl salicylate (MeSA) is involved in plant — plant communication, and recent studies have proved its involvement in systemic acquired resistance in plants (Park et al. 2007).

Effects of plant volatiles on the behavior of forest pests are of both fundamental interest to biologists and great practical interest to foresters (Tominaga et al. 1984; Nordlander 1990, 1991; Byers 1992; Wibe et al. 1997; Thiery and Marion-Poll 1998; Byers et al. 2000; Huber et al. 2000; Bichão et al. 2003; Miller et al. 2003; Asaro et al. 2004). Our results show that some of the volatiles released by mite-infested spruces significantly decreased the attraction of the pine weevil *H. abietis* to host volatiles from spruce. Thus, the biosynthesis of (-)- linalool and MeSA might prevent the trees from being damaged. (*E*)-β-Farnesene was attractive to *H. abietis* when tested alone, but did not affect the attraction of weevils to wounded spruce odor when large amounts of the attractive α-pinene (Nordlander 1990) were released. (*E*)-β-farnesene released from pine has been shown to attract egg parasitoids (Mumm and Hilker 2005), and it might attract *H. abietis* to trees infested by sucking insects. Thus, further tests in the field are needed to determine whether conifer seedlings naturally infested with mites are more or less attractive to the pine weevils than the healthy seedlings.

Minor amounts of aromatic compounds such as benzaldehyde, benzyl alcohol, 1,2- and 1,3-dimethoxybenzene, benzyl acetate, benzoic acid, vanillin, and estragole were detected in the emissions of the mite-infested spruces. The biosynthesis of aromatic compounds related to the scents of many flowering plants has been intensively investigated

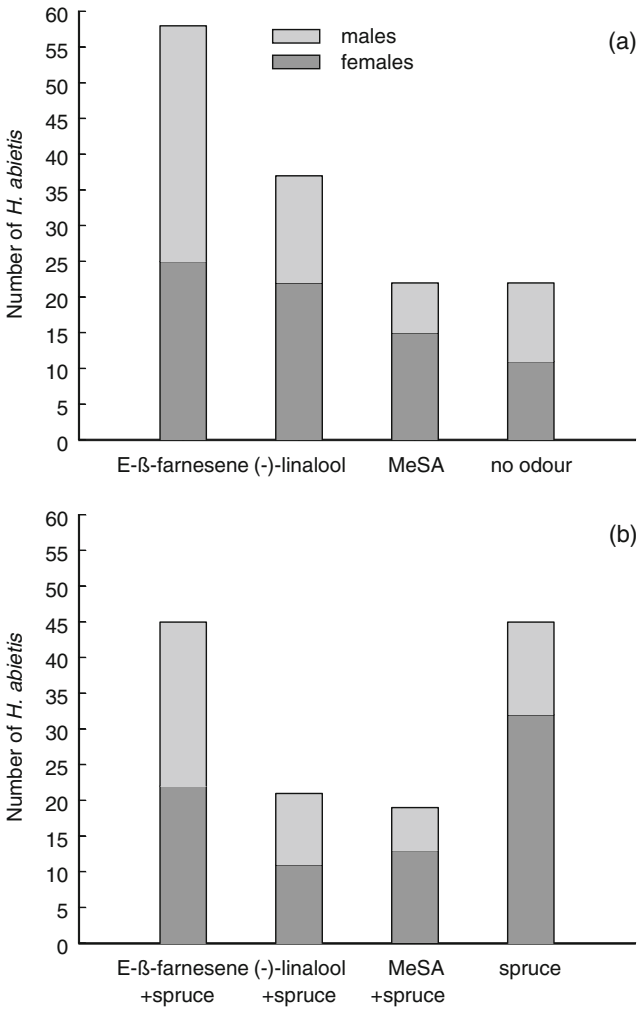

Fig. 3 Number of *Hylobius abietis* weevils entering traps baited with stress-related compounds alone (**a**) or in combination with host odor from Norway spruce twigs (**b**)

(Dudareva et al. 1998, 2000; Dudareva and Pichersky 2000; Guterman et al. 2006; Koeduka et al. 2006). The presence of vanillin, benzaldehyde, benzoic acid, and several unknown aromatic compounds in the spruce volatiles is probably related to lignin biosynthesis or precursors (Schultz 1987; Gang et al. 2001; Dudareva et al. 2006; Jourdes et al. 2007). Similar induction of phenolic compounds by pathogens or fungi, in both pines and spruces, has been observed by several authors (e.g., Campbell and Ellis 1992; Cvikrová et al. 2006). The induced production of small amounts of dimethoxybenzenes is interesting since these compounds are antifeedants for the pine weevil (Borg-Karlson et al. 2006), as are MeSA and structurally related aromatic compounds in sufficiently large concentrations (Legrand et al. 2004; Borg-Karlson et al. 2006). Furthermore, in our recent investigation (Kännaste et al. 2008) we showed that large amounts of MeSA are released from a pine weevil-susceptible spruce clone, and we observed a clear dose-dependent antifeedant effect of MeSA on *H. abietis* in previous studies (unpublished data).

Several biosynthetic pathways are involved in the production of herbivore-induced plant volatiles. The co-occurrence of myrcene and (-)-limonene is consistent with the results of studies by Martin et al. (2004), in which myrcene was identified as a by-product of MeJA-activated limonene synthases. Geraniol and the corresponding aldehyde, geranial, were both found in the emissions. Geranial and neral could be released by the mites (Morita et al. 2004), since in several species they act as alarm pheromones or attractants (Nishimura et al. 2002; Hiraoka et al. 2003).

The diversity in volatiles emitted from the mite-stressed plants reflects the diversity in induced synthase activities. There were no clear differences in the emissions of some terpenes induced by the two mite species, but as shown in the representative chromatograms, the release of (*E*)-β-farnesene seems to be mite specific (Table 1, Fig. 1). The induced terpenes, such as the farnesenes and linalool, and the aromatic compounds like MeSA, indicate that "cross talk" occurs between biosynthetic pathways (Dudareva et al. 2006), especially in the spruces infested with *O. ununguis*, which released larger amounts of MeSA. In the present study, we showed that induced (*E*)-β-farnesene is attractive to pine weevils in the absence of host monoterpenes, while linalool and MeSA are deterrents. However, further studies on the relevance of these compounds as resistance markers are required, using clones with known resistance and susceptibility to various infestations.

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different groups of *Oxytropis* exist, with one group containing swainsonine and the other containing no detectable swainsonine or concentrations that are near the detection threshold.

The objective of this research was three fold: (1) to determine if there are two groups of *O. sericea,* and, if so, the amounts of endophyte in each; (2) to determine the concentration of swainsonine and amount of endophyte in the different plant parts of *O. sericea* at a single developmental stage from a single population; and (3) to compare the concentration of swainsonine and the amount of endophyte from the same plant when separated into stalks (leaves, scape(s), and flowers/pods).

Methods and Materials

Plant Materials Twenty four plants of *O. sericea* Nuttall (Whitepoint locoweed) were collected in July 2007 from the Raft River Mountains, Utah (N 41º 54′ 15.4″ W 113º 20′ 54.9″). Plants collected were in full flower/early pod and divided into their corresponding parts representing the root (tap root), crown (non-photosynthetic woody tissue extending from ground level to tap root), leaves (odd-pinnate with leaflets being opposite), scapes (flowering stem), and flowers/pods. *Oxytropis sericea* has no stem material, and leaves and scapes arise from the crown. These plants were used to investigate objectives 1 and 2. In addition, 10 plants were collected at the same location and time but the plants were divided into 5 equal stalks where each stalk contained leaves, scape(s), and flowers/pods. These plants were used to investigate objective 3. A control sample, representing a composite of a single plant, was extracted to determine the range, mean, and relative standard deviation associated with the method of extraction and analysis as part of objective 3. Immediately after collection, all plant material was bagged and frozen on dry ice. Upon return to the laboratory, plants were freeze-dried and ground through a 2 mm screen in a Wiley mill. Swainsonine and DNA were extracted from this plant material for analyses.

DNA Extraction DNA was extracted from freeze-dried, ground plant material (~20 mg) by using the DNEasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA). All extractions were performed according to the manufacturer's instructions. DNA was quantified with the ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

PCR Primer Design The PCR primers used have successfully detected the presence of the fungal endophyte, *U. oxytropis* in *Oxytropis* and *Astragalus* species (Ralphs et al. 2008). The primers used were ITS 5 (5' GGA AGT AAA AGT CGT AAC AAG G) (White et al. 1990) and OR1 with an additional base pair at the 3' end (5' GTC AAA AGT TGA AAA TGT GGC TTG G 3'), which amplify the internal transcribed spacer (ITS) region. Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA).

Quantification of the Fungal Endophyte For the quantification of the fungal endophyte, a standard curve (30, 10, 3, 1, 0.3, 0.1, 0.03, 0.01 ng fungal DNA) was prepared from DNA extracted from a pure culture of the endophytic fungus (Cook et al. 2009b). Each analysis included three replicate reactions (25 µl) for each DNA quantity. Each analysis also included a non-template control reaction, in which water was substituted for the DNA to confirm that the reagents were free from contaminating template DNA. The equation derived from the standard curve was $y=-3.54x+17.9$, $R^2=0.996$.

Endophyte samples for the standard curves were prepared by analyzing three replicate 25 µl reactions that contained the amount of DNA (0.01–30 ng fungal DNA) indicated on the standard curve. Plant samples were analyzed in three replicate 25 µl reactions that contained 50 ng (5 µl of a 10 ng / µl stock) of total DNA. Each reaction contained 12.5 µl of the QuantiFast SYBR Green PCR Kit master mix (Qiagen Inc., Valencia, CA, USA) and 500 nM each of the forward and reverse primer. Endophyte content (per 50 ng total DNA) was estimated from the endophyte standard curve. Endophyte amounts were expressed as picograms/nanogram total DNA. Samples with endophyte amounts below the quantitative limit of detection of the standard curve were assigned values of 0.2 pg/ng total DNA.

Amplification and detection of fluorescence were performed by using a Bio-Rad CHROMO4 quantitative PCR detector (Bio-Rad Laboratories Inc., Hercules, CA, USA). Thermal cycling conditions comprised an initial denaturation step for 7 min at 95°C, followed by 40 cycles of 15 sec at 95°C, 30 sec at 58°C, 40 sec at 72°C, and a plate read at the end of each cycle. This was followed by a melting profile, to determine the purity of the reaction products, where the temperature was raised from 55°C to 90°C in 0.2°C increments, held for 2 sec at each temperature, and a plate read at each temperature.

PCR products were resolved on a 1% agarose gel containing ethidium bromide at 118 volts for 20 min and visualized under UV illumination. The amplified PCR product was approximately 580 base pairs (bp). PCR products were digested with the restriction enzyme *Ava*II as a diagnostic check since this primer set may also amplify *Alternaria* species, a closely related genus. *Alternaria* species do not contain the restriction site, therefore, a digested product indicates the presence of the locoweed

endophyte, *U. oxytropis*. Restriction enzyme digests contained 5 µl of PCR product, 0.5 µl *Ava*II (New England Biolabs Inc., Ipswich, MA, USA), 1 µl NEB Buffer 4 supplied with the enzyme, and 3.5 µl sterile water for a total volume of 10 µl. Digests were incubated at 37°C for 1.5 h after which the digest was heat inactivated at 65°C for 20 min. Restriction fragments were separated by gel electrophoresis according to the conditions above. A result of two fragments with lengths of 380 bp and 200 bp confirmed the positive amplification of the locoweed endophyte, *U. oxytropis*.

Swainsonine Analysis Samples were analyzed for swainsonine following the procedure described by Gardner et al. 2001. In brief, 100 mg of plant material were extracted with chloroform and acetic acid. The acetic acid portion was passed through a cation exchange resin to retain the swainsonine, which was subsequently eluted with a weak ammonium hydroxide solution. A portion of the final extract then was quantitatively analyzed for swainsonine by LC-MS. The detection limit of swainsonine was 0.001% of dry weight.

Data Analysis Swainsonine and endophyte concentrations were examined in plant parts by using a general linear model of SAS (PROC GLM) (SAS Institute, Cary, NC, USA). Mean comparisons among plant parts after a significant ($P<0.05$) F test was done with preplanned comparisons using the PDIFF procedure in SAS. Initial data analysis revealed two potential groups of *Oxytropis*, one containing readily detectable concentrations of swainsonine and another with little or no swainsonine. Therefore, plants from these two groups were compared for endophyte and swainsonine concentration by using the GLM procedure with least square means for unbalanced sample sizes. Means were compared by using the PDIFF procedure in SAS. The variation in swainsonine concentration among stalks was determined by using descriptive statistics, including the relative standard deviation (RSD, %), which is the absolute value of the coefficient of variation.

Results

Swainsonine in Plant Parts Swainsonine was detected in all plant parts of *O. sericea*. Mean swainsonine concentrations ranged from 0.002% in roots to 0.062% in leaves of *O. sericea* ($N=24$ plants) (Fig. 1). Above ground tissues had higher ($P<0.001$) concentrations of swainsonine than below ground tissues. Swainsonine concentrations did not differ ($P>0.05$) in the leaves, scapes, and flowers/pod or between the root and crown tissues. *Oxytropis sericea*

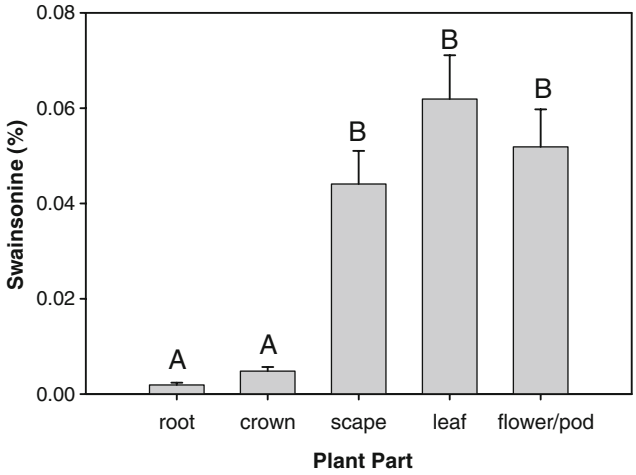

Fig. 1 Swainsonine concentrations (%) differentially accumulate within below ground and above ground parts of *Oxytropis sericea*. Mean swainsonine concentrations±the standard error of parts (root, crown, scape, leaf, and flower/pod) from 24 individual plants. Different letters above each bar represents significance at $P<0.05$

plants appeared to group into two categories, plants ($N=17$) that contained swainsonine in leaves (>0.01%), and plants ($N=7$) where swainsonine was not detected or concentrations that were near the detection threshold in leaves (<0.005%). For further comparison, *O. sericea* plant parts were separated into two categories, group 1 and group 2.

These two apparent groups of *Oxytropis* contained differing concentrations of swainsonine. There was a group x part interaction ($P<0.001$). In group 1 plants, swainsonine was not detected in the root, crown, scapes, and flowers/pods but was detected at a mean concentration of 0.001% in the leaves (Fig. 2). Group 2 plants contained more swainsonine than group 1 plants in all above ground parts ($P<0.001$). Mean swainsonine concentrations in group 2 plants ranged from 0.003% in roots to 0.087% in leaves (Fig. 2). In group 2 plants, above ground tissues had higher concentrations of swainsonine than below ground tissues ($P<0.001$). In addition, in group 2 plants, leaves had greater swainsonine concentrations than flowers/pods and scapes ($P<0.05$).

Endophyte Content in Plant Parts A newly developed qPCR method for *U. oxytropis* allowed determination of endophyte amounts in each of the tissues (Cook et al. 2009b). Endophyte amounts are expressed as pg endophyte DNA/ng total DNA. The endophyte was detected in all plant parts. Mean endophyte amounts ranged from 0.7 pg/ng in the roots to 21.2 pg/ng in the scapes ($N=24$ plants) (Fig. 3). In some instances, endophyte amounts did not mirror swainsonine concentrations in the corresponding tissue type. For example, the crown contained much greater amounts of endophyte compared to its swainonsine content.

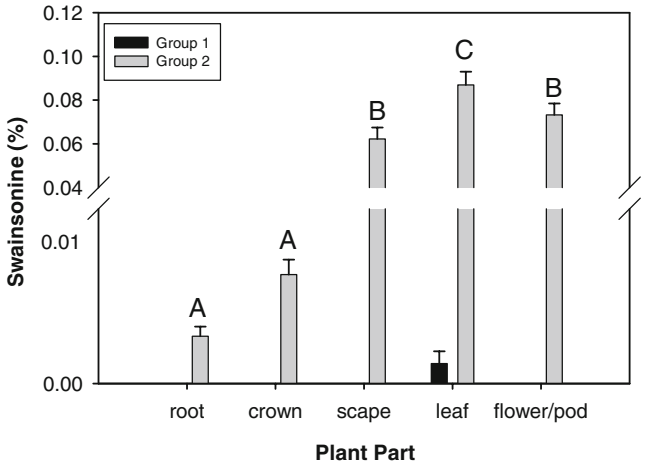

Fig. 2 Swainsonine concentrations (%) differentially accumulate within plant parts and within two groups of *Oxytropis sericea*. Mean swainsonine concentrations±the standard error of parts (root, crown, scape, leaf, and flower/pod) from two groups of *O. sericea* plants that differentially accumulate swainsonine. Group 1=plants that accumulate <0.005% swainsonine in leaves (N=7), Group 2=plants that accumulate >0.01% swainsonine in leaves (N=17). Different letters above each bar represents significance at P<0.05

The apparent groups of *Oxytropis* showed very different concentrations of endophyte (Fig. 4). There was a group x part interaction (P<0.001), as group 1 plants contained less endophyte than group 2 plants in all plant parts but the root. In group 2 plants, the mean endophyte content in the leaves, crown, scapes, and flowers/pods was similar, ranging from 12.1 pg/ng to 29.9 pg/ng. The root was the most different from any tissue type in group 2 plants and contained an average of 0.9 pg/ng endophyte (P<0.001). In group 1 plants, the endophyte was detected in all tissue types; however, the mean endophyte amount ranged from 0.2 pg/ng in the crown to 0.4 pg/ng in leaves (Fig. 4).

Swainsonine in Stalks Five aliquots from a control sample, representing a composite of a single plant, were extracted to determine the range, mean, and relative standard deviation associated with the method of extraction and analysis. The control sample had a mean swainsonine concentration of 0.051% ±0.003 (Relative Standard Deviation (RSD)=6%) (Table 1). To determine if the distribution of swainsonine was uniform within individual plants, five stalks per plant from 10 different plants were analyzed for swainsonine. Swainsonine was detected in 9 of 10 plants (Table 1). Swainsonine was detected in each stalk (N=5 stalks/plant) of the 9 plants that contained swainsonine (Table 1). Based on the results of the control sample analysis, an RSD for swainsonine concentration greater than 6 % for the stalks representing individual plants would suggest that swainsonine is not uniformly distributed throughout the plant. The RSD was greater than 6 % in all plants analyzed, thus swainsonine was not equally distributed within these plants (Table 1).

Endophyte Content in Stalks Five DNA samples were extracted from aliquots of the control sample described in the previous section to determine the range, mean,

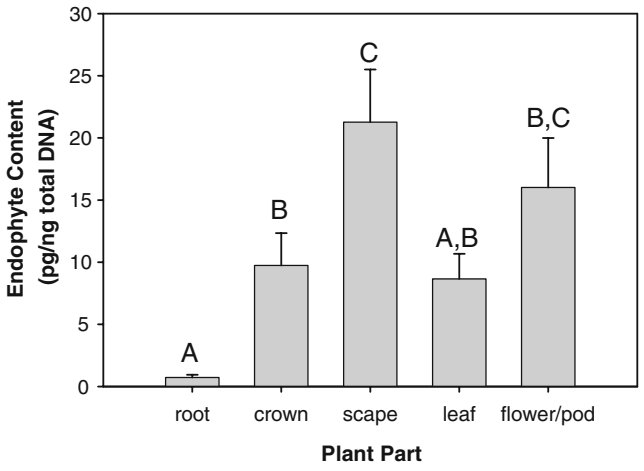

Fig. 3 Endophyte amounts (pg/ng total DNA) differentially accumulate in different plant parts of *Oxytropis sericea*. Mean endophyte amounts±the standard error of parts (root, crown, scape, leaf, and flower/pod) from 24 individual plants. Different letters above each bar represents significance at P<0.05

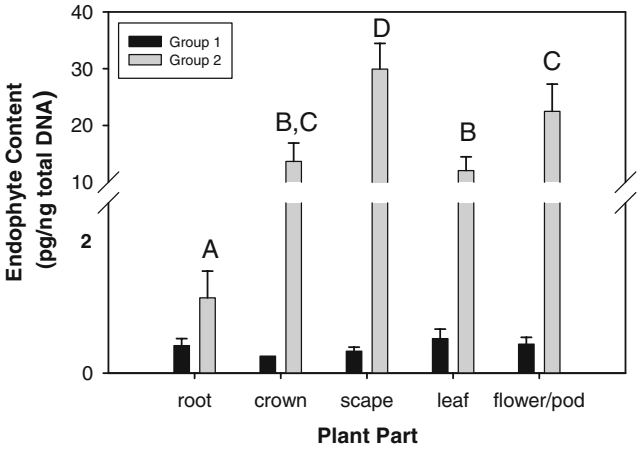

Fig. 4 Endophyte amounts are quantitatively different in two groups of *Oxytropis sericea* plants that accumulate different concentrations of swainsonine. Mean endophyte amounts±the standard error of parts (root, crown, scape, leaf, and flower/pod) from Groups 1 and 2 in Fig. 2. Different letters above each bar represents significance at P<0.05

Table 1 Swainsonine and endophyte distribution within stalks of *Oxytropis sericea* plants

Plant	Swainsonine (%)				Endophyte (pg/ng total DNA)			
	Mean	Range	S	RSD[a] (%)	Mean	Range	S	RSD[a] (%)
1	0.066	0.053–0.082	0.012	18	31.8	26.5–41.6	6.2	19
2	0.056	0.044–0.070	0.011	20	21.3	15.2–31.6	6.6	31
3	0.075	0.064–0.083	0.008	11	14.4	7.2–19.1	4.5	31
4	0.099	0.084–0.116	0.012	12	40.1	23.3–61.4	14.3	36
5	0.056	0.048–0.061	0.005	9	9.8	5.3–14.4	4.1	42
6	0.073	0.018–0.114	0.035	48	9.8	1.6–13.3	5.0	51
7	0.073	0.062–0.089	0.011	15	19.6	5.5–27.9	12.3	63
8	0.068	0.058–0.083	0.011	16	4.9	2.7–5.8	1.3	26
9	0.088	0.075–0.104	0.013	15	13.5	12.4–16.0	1.6	12
10	n.d.				0.2			
Mean (1–9)	0.073		0.013	18	18.4		6.2	34
Control[b]	0.051	0.047–0.54	0.003	6	29.6	23.8–35.8	5.2	17

[a] RSD(%)-Relative Standard Deviation was calculated by dividing the standard deviation by the mean and multiplying by 100

[b] The control, representing a composite of a single plant, represents 5 aliquots that were extracted to determine the range, mean, and relative standard deviation associated with the method of extraction and analysis for swainsonine concentration and endophyte amount

and relative standard deviation associated with the method of extraction and analysis. The control sample had a mean endophyte amount of 29.6 pg/ng±5.2 (RSD=17%). To determine if the distribution of the endophyte was uniform within a plant, 5 stalks per plant representing 10 plants were sampled and analyzed for endophyte amounts. The endophyte was detected in all 10 plants. In addition, the endophyte was detected in every stalk from all ten plants (Table 1). Plant No. 10 was a plant with no detectable swainsonine, and it contained endophyte amounts near or below the quantitative detection limit of 0.2 pg/ng total DNA. Based on the results of the control sample analyses, an RSD for endophyte amount greater than 17 % for the stalks representing individual plants would suggest that the endophyte is not uniformly distributed throughout the plant. The RSD was greater than 17 percent in 8 of the 9 plants. Thus like swainsonine, the endophyte was not equally distributed in the analyzed plants (Table 1).

Discussion

It has long been known that *O. sericea* contains swainsonine (Molyneux and James 1982), and more recent research has shown that swainsonine accumulates to highly variable amounts within any single *O. sericea* population, ranging from not being detected to approximately 0.08% (Gardner et al. 2001). Furthermore, it has been shown that a fungal endophyte, *Undifilum oxytropis*, is responsible for the synthesis of swainsonine (Braun et al. 2003; Pryor et al. 2009). The results of this study confirm these previous observations in addition to: (1) identifying two groups of *O. sericea* and quantifying the amounts of endophyte in each; (2) measuring the concentration of swainsonine and amount of endophyte in the different plant parts of *O. sericea* at a single developmental stage; and (3) determining if the concentration of swainsonine and the amount of endophyte from the same plant were uniformly distributed in individual stalks of the same plant.

By using methods previously developed to quantify swainsonine and a newly developed quantitative PCR method to quantify the endophyte, the concentration of swainsonine and amount of endophyte in the different plant parts of *O. sericea* were investigated at a single developmental stage. Swainsonine and the endophyte were found in all plant parts. Swainsonine concentrations were approximately 10 times greater in above ground tissues than below ground tissues (Fig. 1). Endophyte amounts differed among plant parts and in some instances did not mirror the concentrations of swainsonine in the corresponding tissues (Fig. 3). For instance, the crown contained a comparable amount of endophyte to above ground tissues of the plant, but did not contain a comparable amount of swainsonine. This discrepancy may be explained by swainsonine acting as a mobile secondary compound, and thus being better mobilized, or there is the possibility that the endophyte in the crown tissue is not producing swainsonine. However, irrespective of the low swainsonine concentration, the large amount of

endophyte in the crown may serve as a reservoir for the endophyte for subsequent growth in the following year.

Plants were classified into two groups based upon swainsonine concentration in leaves (Fig. 2). Plants, where swainsonine was not detected or concentrations that were near the detection threshold, occurred at a frequency of approximately 30% within the sampled population of *O. sericea*. Analysis of the qPCR data showed that amounts of endophyte differed between these two groups (Fig. 4). The endophyte, *U. oxytropis*, has been shown to produce swainsonine in *O. sericea*, therefore, these quantitative differences in endophyte amount probably explain the difference in swainsonine concentrations between these two groups of plants. We hypothesize that the observations made for this population of *O. sericea* in regard to endophyte amounts can be applied to other populations of *O. sericea* in Colorado and New Mexico where plants within the same population have been observed to differentially accumulate swainsonine (Gardner et al. 2001; Ralphs et al. 2008).

By using methods to quantify swainsonine and the endophyte, swainsonine concentrations and endophyte amounts were investigated in five stalks from ten plants of *O. sericea*. The mean endophyte content in above ground parts of *O. sericea* containing swainsonine was approximately 18.4 pg/ng total DNA, or a relative endophyte biomass of 1.84% with a range 0.5 to 4.0% (Table 1). In addition, the results show that the concentration of swainsonine and amount of endophyte are not equally distributed within stalks from the same plant (Table 1). These results have implications in regard to sampling techniques for future studies, mainly that sampling the same plant multiple times over a growing season may lead to erroneous conclusions. We recommend that for any sampling regime, the whole plant or all above ground parts be harvested, thus enabling one to make meaningful conclusions in regard to swainsonine and endophyte content.

The perennial ryegrass / fescue endophyte relationship is agronomically important and ecologically important in shaping ecosystems (Rudgers and Clay 2007; Cheplick and Faeth 2009). Our results highlight some similarities and differences between the perennial ryegrass / fescue endophyte relationship and the *O. sericea* endophyte relationship. First, the relative endophyte biomass (0.5 to 4.0%) in *O. sericea* was similar to the relative endophyte biomass in an *Epichloe*/*Neotyphodium* symbiotic assocatiation with perennial ryegrass, estimated to be between 0.3% to 1.9% (Young et al. 2005). Second, the distribution of the endophyte in *O. sericea* plant parts was similar to those reported for tall fescue by Dombrowski et al. (2006) except that, unlike *O. sericea,* the endophyte was not detected in the roots of tall fescue. Third and most critical, in perennial ryegrass and tall fescue, plants are either endophyte positive, thus alkaloid positive, or endophyte minus, thus alkaloid minus. Whereas in *O. sericea*, plants are either endophyte positive, thus swainsonine positive, or the endophyte is detected, but amounts are below a critical threshold, and swainsonine is not detected, or concentrations are near the detection threshold.

The difference in accumulation of swainsonine between the two groups of *O. sericea* leads to the question of the relative toxicity posed by each group. Molyneux et al. (1994) suggested a conservative threshold toxicity of 0.001%. Stegelmeier et al. (1999) showed that doses at or above 0.2 mg/kg body weight/day for at least 21 days produced irreversible neurological disease. Much less is known about lower doses over longer periods of time; however, it has been suggested that it may lead to weight loss and biochemical lesions (Stegelmeier et al. 1999). Swainsonine concentrations of 0.001%, the concentration found in leaves of group 1 plants, would produce a dose of 0.2 mg/kg body weight/day only when locoweed consumption was 100% of their diet at an intake of 2% of their body weight. Therefore, it is unlikely that locoweed plants with swainsonine concentrations of 0.001% would pose a significant risk of causing irreversible neurologic disease; however, grazing animals may incur other reversible negative consequences such as loss of weight.

In summary, *O. sericea* has two groups that differ in their swainsonine accumulation, most likely because of the differences in endophyte amounts between the two groups. These results lead us to hypothesize that *Oxytropis* species in which plants within the same population differ in their swainsonine accumulation also will have differing amounts of endophyte, and we hypothesize that similar results will be found with *Astragalus*. Furthermore, we hypothesize that plants having low or non-detectable swainsonine concentrations may have arisen due to imperfect transmission of a critical amount of the endophyte to the seed or seedling (Afkhami and Rudgers 2008) that is required for effective colonization by the endophyte. Alternatively, low swainsonine-containing plants may suppress endophyte growth due to a plant genotype by endophyte interaction (Adcock et al. 1997; Roylance r. 1994). These hypotheses merit further investigation. In addition, future research is necessary to determine if there are differences in fitness, as observed in grass-endophyte relationships (Rudgers and Clay 2007; Cheplick and Faeth 2009), between plants belonging to these two groups of *O. sericea*.

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tion), and PDMS tubing. The PDMS sorbents were placed in the pots at planting and removed at harvests 29, 55, and 88 days after planting. As analyzed by HPLC, the amount of sorgoleone recovered increased with time. Barto and Cipollini (2009) used 10 cm lengths of PDMS tubing (buried in soil, then recovered and extracted) in an attempt to measure allelochemicals beneath garlic mustard, *Alliaria petiolata*, and were able to detect low amounts of one flavonoid, isovitexin-6″-O-β-D-glucopyranoside. Weidenhamer et al. (2009) used solid phase root zone extraction (SPRE) probes constructed by inserting stainless steel wire into PDMS tubing to recover nano- to microgram quantities of thiophenes from soil beneath growing marigolds, using a 24 hour sampling time.

PDMS-based materials are used widely for trace analysis of anthropogenic contaminants. They are used as stationary phase in capillary columns for the analyte separation in gas chromatography, and as enrichment material in several analytical techniques that include solid phase microextraction (Arthur and Pawliszyn 1990), stir bar sorptive extraction (Baltussen et al. 1999), and thin-film microextraction (Bruheim et al. 2003). PDMS also has been applied for diffusive sampling in sediment and soil (Mayer et al. 2000; Weidenhamer 2005; Weidenhamer et al. 2009) and for passive sampling in the aquatic environment (e.g., Cornelissen et al. 2008). Recently developed techniques are based on the permeation of non-polar analytes through the wall of PDMS microtubing: Ooki and Yokouchi (2008) constructed a silicone membrane tube equilibrator from PDMS tubing for the sea water to gas equilibration of volatile organic compounds; Mayer et al. (2009) used 6 m PDMS microtubes as silicone membrane equilibrator to measure polycyclic aromatic hydrocarbons directly in tissue and oil samples. The tubing was immersed in the sample, and compounds were extracted by pushing a plug of methanol through the tubing and collecting it in an HPLC vial for analysis. In the present study we apply PDMS microtubing for the in situ sampling of root exudates and the subsequent elution into methanol while keeping the tubing in place.

The objective of the experiments described in this paper was to compare the ability of three PDMS-based techniques to measure the spatial and temporal dynamics of thiophenes in the root zone of African marigold, *Tagetes erecta* L. Solid phase root zone extraction probes were used to monitor thiophene concentrations at different depths beneath marigolds grown in PVC pipes. Marigolds were grown between foil-lined glass plates, and thiophene distributions in the soil were measured by two techniques: (a) PDMS sheets were press-applied to the roots and analyzed after 24 h; and (b) PDMS microtubing (1 m lengths) was placed over the roots and sampled at 24 h intervals by flushing 95% methanol through the tubing. Because PDMS selectively sorbs lipophilic compounds (Baltussen et al. 1999), marigold was considered to be a good model species for these studies due to the highly lipophilic character of the thiophenes produced by marigold roots (Fig. 1, Bohlmann et al. 1973; Downum and Towers 1983). In addition, these thiophenes have high biological activity against a number of organisms (Bakker et al. 1979; Campbell et al. 1982), and have been reported to be released into the marigold root zone (Campbell et al. 1982; Tang et al. 1987; Martin and Weidenhamer 1995; Weidenhamer et al. 2009).

Methods and Materials

Growth Media and Plant Cultivation Conditions Plants were grown in a greenhouse under natural sunlight during June – July 2009. In general, daytime temperatures ranged from 21–32° C and nighttime temperatures from 16–22° C. A 1:1 (v:v) mixture of sand and a peat/vermiculite-based growth medium (Redi-earth® plug and seedling mix) was used for all experiments. Plants (*Tagetes erecta* cv. 'Crackerjack mix' and *Tagetes patula* cv. 'Janie primrose') were watered as needed, and received weekly applications of a general purpose fertilizer solution (Peters Professional® all purpose plant food, 24-8-16 plus B, Cu, Fe, Mn, Mo, and Zn). Fertilization was not done immediately before or during sampling.

SPRE Probe Study Marigolds (*T. erecta* cv. 'Crackerjack mix', thinned to 3 plants per pipe) were grown in four 7.5 cm ID, 44 cm tall PVC pipes. The pipes were mounted on a flat, circular base. A 10 cm layer of gravel at the base and three drainage holes drilled at the bottom kept the pots well-drained. Three evenly spaced holes (1.5 mm diam, 5 cm apart) were drilled at eight depths (2.5 cm intervals, from 2.5–20 cm below soil surface) to allow access for sampling. Pipes were wrapped with polyethylene film to

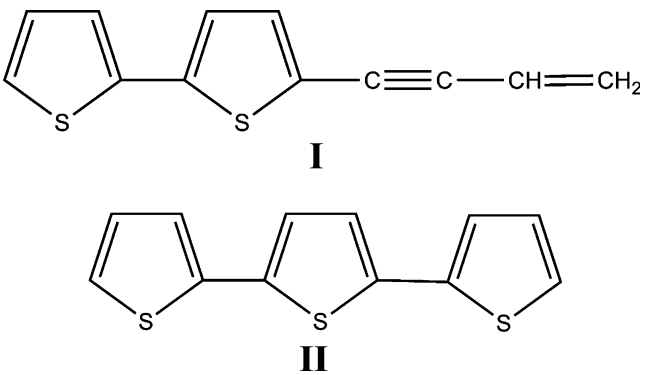

Fig. 1 Structures of 5-(3-buten-1-ynyl)-2,2′-bithienyl (BBT, I) and α-terthienyl (2,2′:5′,2″-terthiophene, α-T, II)

prevent the drainage of water through the probe access holes.

SPRE probes were prepared as described in Weidenhamer et al. (2009). Stainless steel wire (22-gauge) was inserted into 5 cm lengths of hexane-swelled Silastic® tubing (0.64 mm ID×1.19 mm OD, Fisher Scientific catalog no. 11-189-15B). The probes were sequentially cleaned with hexanes, dichloromethane, methanol, and water by soaking for 10 min in each, and then dried in an oven at 70°C. Once the probes were dried, they were stored in tightly capped vials until use.

At 3, 4, and 5 wk after planting, SPRE probes were inserted into the 96 probe access holes (3 access holes× 8 depths×4 pipes) and left in place for 24 h. The 3 probes at each depth were pooled, and thiophenes were extracted from the probes by first rinsing with water to remove adhering soil particles, blotting dry, stripping the tubing from the wire support, and sonication in an amber autosampler vial with 700 µl of 95% methanol (methanol: water 95:5, v:v) for 12 min.

Glass Box PDMS Sheet Study Glass boxes were constructed by using two 20x20 cm glass plates (0.3 cm thickness). Four bottle caps were glued in the corners of one plate to maintain a 1 cm spacing between the plates. Plates were covered with non-stick aluminum foil (to protect light-sensitive thiophenes from degradation), sealed with polyethylene film on three sides, and held together with clamps before filling with growth medium. Drainage holes allowed removal of excess water. Marigolds (*T. erecta* cv. 'Crackerjack mix') were thinned to two seedlings per box.

Three-five wk after planting, boxes were unclamped and a 17.5×20 cm sheet of clear PDMS (0.8 mm thickness, obtained from Specialty Silicone Products, Ballston Spa, NY, USA) was press-applied to the root zone. To insure that PDMS sheets were clean, they were soaked in methanol for at least 24 h and then dried at 70° C before application. The glass plate was replaced, the box clamped, and the sheet was left in place for 24 h. PDMS sheets were then washed to remove adhering soil and immediately cut into fifty-six 2.5×2.5 cm segments. Each segment was placed in a vial with 3 ml of 95% methanol and sonicated for 12 min to extract thiophenes.

Glass Box Microtubing Study A glass box with established marigolds (*T. erecta* cv. 'Crackerjack mix') was sampled by using five replicate 1 m lengths of PDMS Silastic® microtubing (0.30 mm ID×0.64 mm OD, Fisher Scientific catalog no. 11-189-14). Prior to use, microtubing was cleaned by soaking in methanol for 24 h and dried at 70° C. One piece of microtubing was coiled over a 4 cm width× 17.5 cm depth section of the root zone (a total of 5 pieces over the 20 cm wide box), with the ends of the tubing left exposed above the soil surface to allow repeated sampling (Fig. 2). Microtubing was sampled by pushing 500 µl of 95% methanol through each tube at a rate of 1 ml/min followed by a 500 µl bolus of air to extrude any residual solvent. The resulting extract was collected for HPLC analysis as it exited the tubing.

The operative range of the silicone tube microextraction method was tested by preparing 1:1 sand:growth medium mixtures spiked with known concentrations of α-Terthienyl (2,2′:5′,2″-terthiophene) (α-T) (Sigma Aldrich). α-Terthienyl was applied in hexane to sand, the hexane allowed to evaporate, and the spiked sand used to prepare sand:growth medium mixtures containing concentrations of 0–10 µg g^{-1}. In a Petri dish, one microtube was coiled over 10 g sand: growth medium, covered, and a second microtube was coiled and then covered with sand:growth medium. A total of 43 g of 1:1 sand:growth medium was used per dish. Holes were drilled in the top of the Petri dish to provide access for sampling. Water was added to bring the dishes to 20% moisture content, and microtubing was sampled at 24, 48, and 72 h (using separate dishes for each sampling time).

Root Dissection In order to gain insight into the possible reasons for the tremendous heterogeneity of measured thiophene concentrations seen in the SPRE probe and glass box PDMS sheet studies, roots of several *T. erecta* and *T. patula* seedlings were dissected and analyzed for thiophene content. Primary and secondary roots and root tips of several seedlings grown in a 1:1 mixture of sand and growth medium were analyzed by extraction in methanol. Two week old plants (5–6 cm in length from root cap to cotyledon) were separated from the soil by submersion in

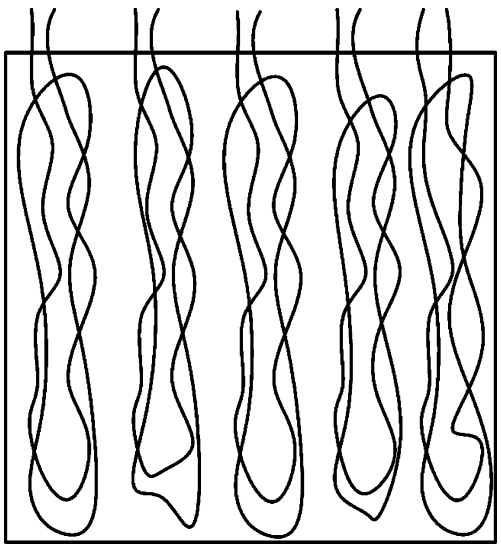

Fig. 2 Schematic diagram showing placement of five 1 m length of PDMS microtubing across the marigold root zone in a 20 cm wide glass box

distilled water. Growth medium was carefully washed from the roots. Root hairs (secondary roots) were separated from the main root with a scalpel and forceps. Roots were transferred to a Petri dish that contained distilled water, blotted dry on KimWipes®, and weighed on a microbalance to obtain a fresh root mass.

Diffusion Studies For the diffusion studies, SPRE probes loaded with known amounts of α-T were prepared by taking freshly prepared probes and placing them in sand spiked with a known amount of α-T (20–100 ppm) and moistened to 12.5% moisture content. Probes were incubated for 1, 2, 4, or 7 d, and 4 probes were analyzed at each time point to determine the amount of loading. The amount of α-T loaded on the probes increased with concentration and time (data not shown) up to a maximum of 29 μg after 7 d incubation in 100 ppm sand. In subsequent studies, a loading time of 4 d typically was used, and several probes from each batch were analyzed to verify the α-T content.

Loaded SPRE probes (containing 22 μg α-T) were used to measure the diffusion of α-T through sand and a 1:1 mixture of sand and growth medium. A loaded probe was placed in a Petri dish containing the sand or growth medium maintained at 12.5% moisture content, and a blank probe placed 1–5 cm away. After 7 d, both probes were extracted and analyzed for α-T content.

HPLC Methods A standard sample of α-T was obtained from Sigma Aldrich. 5-(3-buten-1-ynyl)-2,2′-bithienyl (BBT) was obtained by bulk extraction of marigold roots and preparative TLC following methods of Downum and Towers (1983), and its identification confirmed by GC-MS (data not shown). The HPLC system was an Agilent 1100 Series equipped with a binary gradient pump, autosampler, and diode array detector. Separation was carried out on an Ultra C18 5 μm particle size column (150×4.6 mm), with detection at 360 nm. BBT and α-T were identified based on both retention time and UV spectra using the diode array detector. Analysis of unused PDMS materials verified that these were free from contaminants when extracted and analyzed by HPLC. BBT was quantified based on the ratio of the molar absorptivity of BBT to that of α-T at 360 nm. Detector response was linear for α-T over a concentration range of 0.05–50 mg L^{-1}. The quantitation limit was below 2 ng thiophene injected. The mobile phase was 95:5 methanol: water (v:v), at a flow rate of 1 mL min^{-1}. All PDMS extracts were analyzed within 24 h of extraction, and were stored in amber autosampler vials to prevent degradation from light exposure. PDMS samples that could not be analyzed immediately were stored in a freezer (−20° C) until extraction and analysis. All samples were syringe filtered (0.22 μm nylon filter) prior to analysis, and injected in duplicate with the two runs averaged. Typical run to run variation was less than 1%. The injection volume was 25 or 75 μL depending on sample size.

Results and Discussion

SPRE Probe Study Results of analyses of SPRE probes placed at varying depths beneath marigolds grown in PVC pipes are shown in Tables 1 and 2. There was tremendous variability in the amounts of α-T and BBT recovered. Table 1 shows recovery as a function of depth at 3 weeks after planting, and it can be seen that the standard deviations approach or exceed the means for all values. High probe to probe variability also was seen in studies with *Tagetes patula*, in which SPRE probes were inserted into soil around marigolds growing in outdoor garden beds (Weidenhamer et al. 2009). This variability was reduced somewhat by pooling multiple probes to obtain a more integrated sample, but standard deviations in the previous study still were above 50% of the mean values measured even when three probes were combined (Weidenhamer et al. 2009).

When the total amounts of α-T and BBT recovered from each pipe at the three samplings are compared, high variability is also seen (Table 2). For example, at the first sampling 3 weeks after planting, the total amount of α-T recovered (from 24 SPRE probes) ranged from 280 to 3,170 ng, and the total amount of BBT recovered ranged from 870 to 8,150 ng. There also was variability from one sampling to another, as seen by the fact that pipe 1 yielded the most α-T at the first sampling, but the least α-T at the next sampling. While overall the average amount of BBT found was four times that of α-T, the amounts found in individual pipes at a given sampling sometimes deviated

Table 1 Recovery of thiophenes as a function of depth at 3 weeks after planting, using SPRE probes to sample marigolds grown in PVC pipes at depths of 2.5–20 cm. Mean thiophene recovery is expressed in ng per SPRE probe ± standard deviation

Thiophene content as function of depth at 3 weeks after planting		
Depth, cm	α-T, ng per probe ± SD	BBT, ng per probe ± SD
2.5	277±385	87±107
5	72±72	44±37
7.5	37±54	29±25
10	126±190	336±686
12.5	39±53	61±48
15	25±31	33±31
17.5	18±11	25±20
20	56±55	289±371

α-T = α-Terthienyl (2,2′:5′,2″-terthiophene)

BBT = 5-(3-Buten-1-ynyl)-2,2′-bithienyl

Table 2 Recovery of thiophenes using SPRE probes to sample marigolds grown in PVC pipes. Total ng of thiophene recovered in each pipe at each sampling date is shown. A total of 24 probes were used per pipe per sampling date

Pipe	α-T, total mass per pipe, ng	BBT, total mass per pipe, ng	Total thiophene per pipe, ng
Three weeks after planting			
1	3170	870	4040
2	1210	8150	9360
3	3140	3280	6420
4	280	1250	1530
Four weeks after planting			
1	140	1720	1860
2	210	2760	2970
3	1320	9890	11210
4	260	2380	2640
Five weeks after planting			
1	184	1680	1860
2	298	4970	5270
3	1010	7250	8260
4	1110	5610	6720
Overall mean per pipe±s.d.	1030±1090	4150±2990	5180±3240

α-T = α-Terthienyl (2,2′:5′,2″-terthiophene)

BBT = 5-(3-Buten-1-ynyl)-2,2′-bithienyl

widely from that proportion, as seen with pipes 1 and 3 at the first sampling.

Glass Box PDMS Sheet Study PDMS sheets successfully recovered both α-T and BBT, but the amounts found were highly variable as found in the PVC pipe study (Table 3). Two boxes were sampled once approximately 3 weeks after planting. Immediately after sampling, the soil in each box was sectioned into a grid corresponding to the 2.5×2.5 cm^2 sections of the PDMS sheet, and analyzed for either total thiophene content (box 1) or total root mass (box 2). No correlation was found between either total thiophene content or root mass and the amounts of thiophenes measured per PDMS section (data not shown). A third box was sampled twice, at 4 and 5 weeks after planting. As can be seen from the results summarized in Table 3, very different overall results were obtained at each sampling. The maximum amount of α-T recovered on one of the 2.5×2.5 cm^2 sections was 5,990 ng (bordered by sections all yielding less than 40 ng), while BBT concentrations were less extreme in their variation, with a maximum amount of 260 ng recovered (bordered by sections containing 85–216 ng).

Glass Box Microtubing Study The final approach used to measure thiophenes in the marigold root zone was to place several 1 m long coiled PDMS microtubes over portions of the marigold root zone. The silicone tube microextraction approach allows repeated sampling of root exudates without further disturbance of the soil, and experimentally is quite simple, with samples collected directly for HPLC analysis by passing 95% methanol through the microtube. The results of our first applications of silicone tube microextraction to measure thiophenes were remarkably different from those seen with other sampling methods (Fig. 3). The amounts of both BBT and α-T recovered both were highest at the initial sampling, and BBT was higher in the middle and one side of the plate, while α-T recovery was uniform across the plate (Fig. 3). Recovery of BBT slowly dropped from days 2–6, while amounts of α-T recovered and stabilized quickly at approximately 11–14 ng. While the recovery of BBT remained fairly variable among replicate microtubing extractors, with relative standard deviations of 63–87% of the mean values, recovery of α-T with the microtubing was more uniform, with relative standard deviations of 4.0–8.4% of the mean values (Table 4).

The operative range of the silicone tube microextraction was tested for α-T by spiking 1:1 sand:growth medium mixtures with known amounts of α-T and incubating microtubing in the spiked soil for 24 h. This yielded a highly linear response over the range of 0–10 ppm α-T (Fig. 4), and additional measurements (data not shown) show that response was linear to 800 ppm. These data imply that recoveries of 11–42 ng α-T from silicone tube microextractors in the marigold root zone correspond to soil concentrations well below 1 ppm α-T. Separate spiked Petri dishes sampled at 48 and 72 h also yielded linear responses, with slightly increased slopes for each 24 h period. However, repeat sampling of the 24 h dishes at 48 h yielded a lower slope, suggesting that the α-T removed by sampling at 24 h was not completely replaced by diffusion over the next 24 h. Given that sorption to different soil components will vary with time and affect the partitioning behavior of thiophenes, calibration to actual soil concen-

Table 3 Recovery of thiophenes from PDMS membranes press-applied to soil of marigolds grown in glass boxes. Analyses were performed 3, 4 and 5 weeks after planting (WAP). Membranes were analyzed in 2.5×2.5 cm^2 segments, and total thiophene content of the 17.5 cm×20 cm membrane is reported here

Glass Box	α-T, ng per membrane	BBT, ng per membrane	BBT/α-T ratio
1 (3 WAP)	9087	88.8	0.010
2 (3 WAP)	1057	4325	3.09
3 (4 WAP)	517	1965	3.80
3 (5 WAP)	2982	329	0.11

α-T = α-Terthienyl (2,2′:5′,2″-terthiophene)

BBT = 5-(3-Buten-1-ynyl)-2,2′-bithienyl

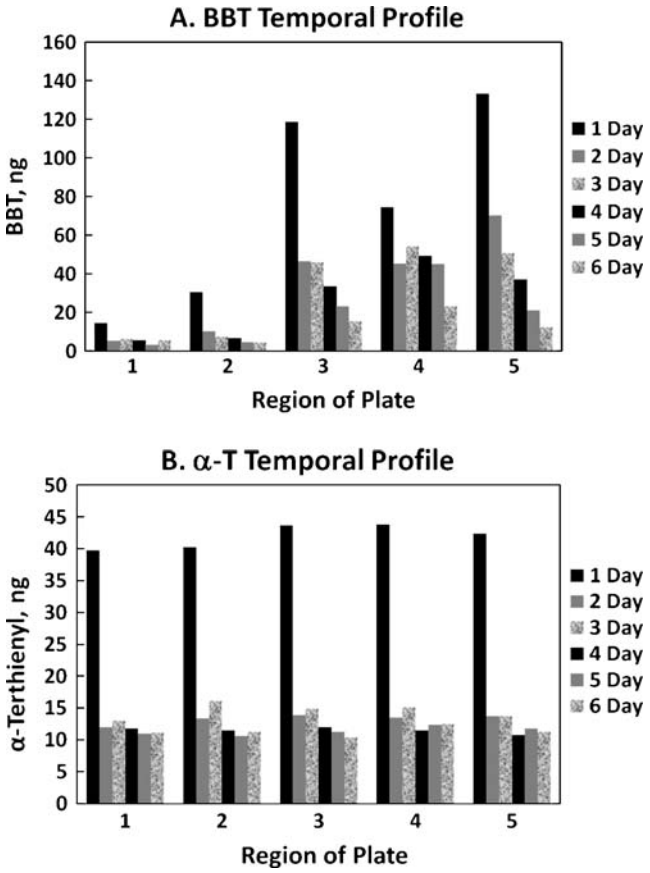

Fig. 3 Amounts of (**a**) 5-(3-buten-1-ynyl)-2,2′-bithienyl (BBT) and (**b**) α-terthienyl (2,2′:5′,2″-terthiophene) (α-T) recovered from five replicate 1 m lengths of PDMS microtubing coiled over successive 4 cm wide×17.5 cm deep sections of marigold root zone across a 20 cm wide glass box. Samples were taken daily for six days. Means and standard deviations for each day are presented in Table 4

trations would be difficult. We envisage two strategies for the calibration of the silicone tube microextraction. (1) The silicone tube can be equilibrated following the principles of equilibrium sampling devices (Mayer et al. 2003), and will then yield measurements of freely dissolved concentrations, chemical activities, or fugacities (Reichenberg and Mayer 2006). This approach will require high surface to volume ratios of the microtubing, and it will be suited for analytes that can be equilibrated within minutes, hours, and possibly days, which appears difficult to achieve for the thiophenes of the present study. (2) Alternatively, the extraction can be operated in the kinetic mode, and it will then yield measurements of the diffusive flux into the silicone similar to the principles of Diffusive Gradients in Thin Films (DGT) that is well established for the sampling of cationic metals (Zhang et al. 1995, 2001).

In follow-up experiments, the effectiveness of the methanol extraction of the tubing was tested by pushing ten 500 µl portions of 95% methanol through tubing incubated in a spiked sand:growth medium mixture, and then extracting remaining α-T from the tubing by sonication in excess 95% methanol. The first wash recovered approximately 40% of the total α-T on the tube, and amounts recovered by subsequent washes followed an exponential decrease, with less than 1% recovered on the tenth wash. After ten washes, less than 5% of the total α-T remained on the tubing. Further work will be required to optimize the methanol extraction procedure and there again are two different strategies. (1) The methanol volume can be minimized to e.g., 100 µl in order to avoid analyte depletion of the silicone, and this will yield the highest possible analyte concentrations in the methanol ($C_{MeOH} = C_{PDMS} * K_{MeOH,PDMS}$) (Mayer et al. 2009). (2) Alternatively, the methanol volume can be increased to several ml aiming for a complete extraction of the PDMS. Optimization of both the sampling step (soil to silicone) and of the extraction step (silicone to methanol) should be feasible, and further studies aiming at a well defined calibration of silicone tube microextraction are underway.

Root Dissection and Diffusion Studies Thiophene content of roots was variable, with no clear pattern in variation from primary to secondary roots (data not shown). Concentrations of BBT and α-T in *T. patula* roots ranged from 0.22–1.38 µmol g^{-1} fresh weight and 0.01–0.04 µmol g^{-1} fresh weight, respectively. Concentrations of BBT and α-T in *T. erecta* roots were lower, ranging from 0.09–0.17 µmol g^{-1} fresh weight and 0.01–0.02 µmol g^{-1} fresh weight, respectively. These data are similar in magnitude to literature reports of thiophene concentrations in marigold roots. Croes et al. (1989) reported total thiophene concentrations of 0.22–0.41 µmol g^{-1} in attached *T. patula* roots, while Croes et al. (1994) found 0.77 µmol thiophenes g^{-1} in roots of six-day-old seedlings of *T. patula*. Jacobs et al. (1994) found total thiophene concentrations in *T. patula* roots to be consistently higher than those of *T. erecta*, which they reported to range from 0.14–0.21 µmol g^{-1}.

Table 4 Mean amounts (± SD) of α-T and BBT measured over six days from five replicate 1 m lengths of PDMS microtubing placed over 4 cm×17.5 cm sections of marigold root zone in a 20 cm wide glass box. Values of individual replicates are presented in Fig. 3

Day	α-T, ng per microtube	BBT, ng per microtube	BBT/α-T ratio
1	42.0±1.9	74.4±52.3	1.77
2	13.3±0.8	35.6±27.2	2.67
3	14.6±1.2	33.0±24.1	2.26
4	11.5±0.5	26.4±19.4	2.29
5	11.4±0.7	19.5±17.0	1.71
6	11.3±0.8	12.2±7.7	1.08

α-T = α-Terthienyl (2,2′:5′,2″-terthiophene)

BBT = 5-(3-Buten-1-ynyl)-2,2′-bithienyl

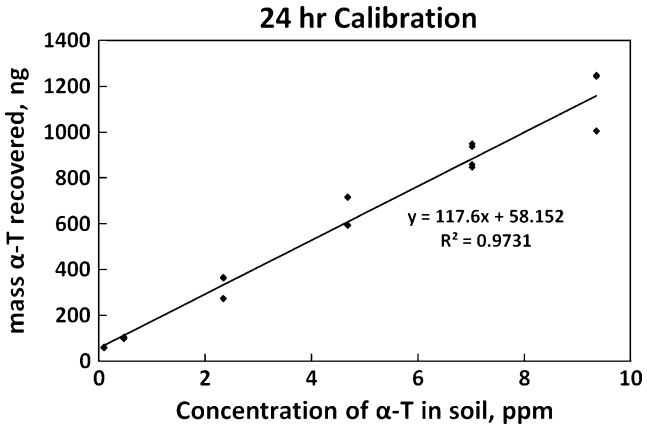

Fig. 4 External calibration of silicone microtubing prepared by spiking 1:1 sand:growth medium with known concentrations of α-terthienyl (2,2′:5′,2″-terthiophene) and incubating 1 m lengths of microtubing for 24 hr

The amounts of BBT found in roots are in general much higher than α-T. For *T. erecta*, the ratio of BBT to α-T ranged from 3.7:1 to more than 20:1 depending on the sample, considerably higher than the typical ratio of BBT to α-T recovered with the various PDMS extraction methods (Tables 3 and 4), suggesting that α-T is either preferentially exuded by roots or is more stable in soil than BBT. Further study will be required to answer this question.

Over seven days, limited diffusion of α-T was observed in sand, with ng quantities of the thiophene detected up to 4 cm from the source probe (Fig. 5a). Slightly greater diffusion was observed in the sand:growth medium mixture, but was still in the ng range compared to the 22 μg loading on the probes (Fig. 5b). Such a result was expected for these highly lipophilic compounds. The limited ability of α-T to diffuse in soil implies that it will tend to stay where it is produced, and indicates that direct contact with marigold roots or rhizosphere soil will be necessary for thiophenes to be available to target plants or organisms.

Taken together, our results provide some evidence for continued release of thiophenes from marigold roots. The limited ability of α-T to diffuse in soil, combined with the decreased amount of α-T recovered when spiked soils were resampled in the microtubing calibration study, implies that the stable concentrations of α-T measured over days 2–6 with the microtubing reflect continued release of α-T into the root zone. However, further studies will be required to verify this.

Comparison of PDMS Sampling Methods The three PDMS-based methods gave markedly divergent results, with much lower variability in amounts recovered seen with the microtubing, especially for α-T. The three techniques are not equivalent in their potential for measuring thiophene distributions in the marigold root zone. The SPRE probes (5 cm length, 1.19 mm outer diam) have an exposed surface area of approximately 1.9 cm^2, and gather data for specific, separate regions of the root zone. The PDMS sheet provides data on a vertical slice of the root zone, providing an average value over each 2.5 cm square (= 6.25 cm^2) segment. The PDMS microtubing (1 m length, 0.64 mm outer diam) has an exposed surface area of approximately 19 cm^2 (accounting for 2 cm at each end of the tube not in contact with soil), and thus averages thiophene concentrations over a larger region of soil than the other two methods. The question of whether the heterogeneity of thiophene distributions observed with the SPRE probe and PDMS sheet methods is real or an artifact of the sampling methods is crucial, given that such variation in the soil could have profound impacts on biological activities of these compounds.

We interpret our data to support the conclusion that the extreme heterogeneity of thiophene concentrations observed with the SPRE probes and PDMS sheets is an artifact of the sampling method and probably due to root

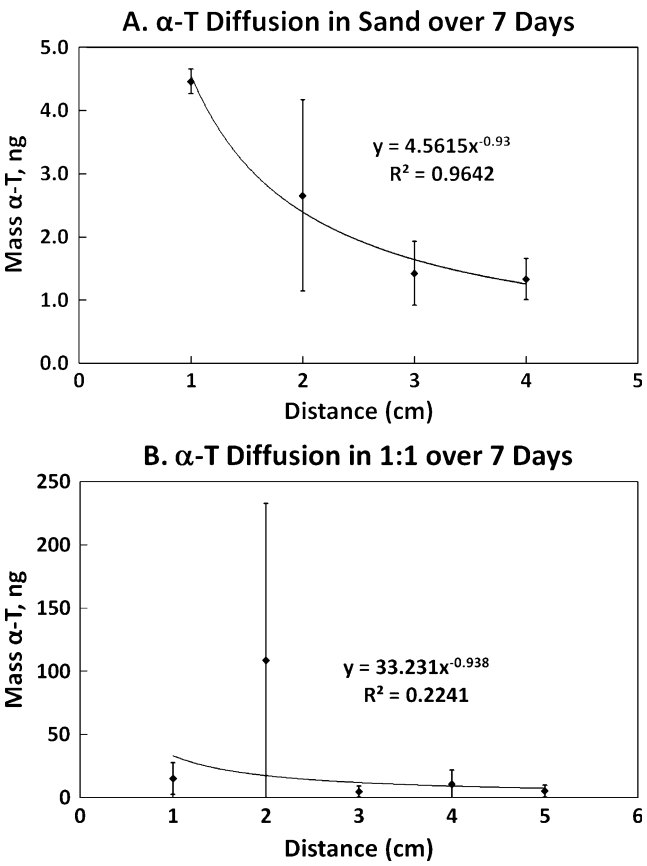

Fig. 5 Diffusion of α-terthienyl (2,2′:5′,2″-terthiophene) α-T from loaded SPRE probes in (**a**) sand and (**b**) a 1:1 sand:growth medium mixture over seven days

injury and/or disturbance by these methods. We base this conclusion on several observations: (a) Variability of the SPRE probe method was extremely high even if the amount of thiophenes found on all 24 probes per sampling was summed (Table 2). The total exposed surface area of 24 SPRE probes is 45.6 cm^2, more than double that of the 1 m microtubes, yet variability was very high for the SPRE method in comparison to the microtubes; (b) Heterogeneity of soil concentrations could be due to localized interactions of roots with soil microbes and microfauna, but as noted above there was no correlation between thiophene recovery and marigold root mass in the PDMS sheet study; (c) The higher recovery of thiophenes on the initial day following application of the microtubing, which was less disruptive than the press-application of PDMS sheets, also seems to underscore the importance of minimizing soil disturbance during sampling, at least for this system. A crucial requirement for obtaining accurate data on allelochemical concentrations in soil is that those dynamics not be altered by the attempt to measure them.

Finally, while PDMS in the form of SPRE probes may not be useful as originally envisioned for measurement of allelochemical dynamics (Weidenhamer et al. 2009), loaded probes may prove useful as a means to deliver known amounts of lipophilic compounds for bioassay (e.g., Mayer and Holmstrup 2008; Kwon et al. 2009).

Advantages and Potential Applications of the Silicone Tube Microextraction Method The silicone tube microextraction method is a further development of the silicone membrane equilibrator technique, which was developed and applied for measurements of volatile organic compounds in seawater (Ooki and Yokouchi 2008) and of polycyclic aromatic hydrocarbons in vegetable and fish oils and mussel tissue (Mayer et al. 2009). In this paper, we call this method silicone tube microextraction as a more descriptive name for placing silicone microtubing for sampling in the root zone. This method has several key advantages for studies of root exudates: (a) Once microtubing is in place, the root zone can be repeatedly sampled without further disturbance; (b) the sampling method is quite simple, and the materials are inexpensive; (c) the method is sensitive to daily and spatial variations in allelochemical content; and (d) calibration of results to available analyte concentrations seems feasible, though this requires further research. The technique should be broadly applicable to the measurement of non-polar root exudates, providing a means to test hypotheses about the role of root exudates in plant-plant and other interactions. It should be possible to place microtubing in pots prior to seeding plants to monitor allelochemical dynamics over time or plant response to treatments such as herbivory, nutrient stress, or competition. Applications of microtubing for field studies also should be possible. Further explorations of the potential of this method for allelochemical analysis in soil are underway.

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produced by a great variety of plant species. In addition, considerable diversity is often observed in the structure and concentration of chemical constituents within exudates of various species or cultivars (Bertin et al. 2003a).

Root exudates and the allelochemicals they contain generally are thought to degrade fairly rapidly within the soil rhizosphere, through the activity of soil microbes, chemical breakdown and immobilization caused by irreversible binding to organic matter and lignin (Blum et al. 1991; Weston and Duke 2003; Weidenhamer 2004). To further characterize allelochemical interference in the rhizosphere, the accumulation of allelochemicals at phytotoxic levels and their fate, persistence, and interactions in soil, are important to examine. For example, the allelopathic activity of black walnut (*Juglans nigra* L.) has been observed for years (Massy 1925; Davis 1928). Some believe that because juglone is sensitive to biotic and abiotic degradation in soil, it is improbable that it persists at phytotoxic concentrations high enough to result in growth inhibition (Schmidt 1988, 1990). Others have argued that the allelopathic potential of juglone is due to its existing concentration in the soil suspension, its renewal rate by release from living roots, and the sensitivity of surrounding species that are impacted by juglone itself (Williamson and Weidenhamer 1990). As allelochemicals undergo chemical and biological degradation upon soil entrance, their accumulation and concentration may likely change rapidly over time in soil settings. Hence, many researchers have expressed their concerns regarding the relative unpredictability of laboratory bioassays in terms of explaining field observations (Inderjit and Dakshini 1995, 1999; Inderjit and Weston 2000).

We have shown in recent studies that m-tyrosine, an analogue non-protein amino acid, is exuded by the living root system of certain fine leaf fescue species. Interestingly, it possesses potent weed suppressive activity and appears to impact plant growth mainly from initial inhibition of plant root growth or radicle elongation at very low concentrations (Bertin et al. 2003a, b, 2007). The laboratory bioassays we utilized for our initial isolation and characterization of *m*-tyrosine were simple assays that evaluated seed germination and radicle elongation in soil-less Petri dishes, with a variety of indicator species. In an attempt to examine further its activity under controlled laboratory conditions before studying its release in rhizospheres of living roots under field conditions, we evaluated its effect upon plant growth in a series of soil-based bioassays, using lettuce (*Lactuca sativa*) L. as a sensitive indicator species. Here, we report on the effects of soil texture upon allelochemical activity as well as soil amendment with nitrogen and activated carbon, in an attempt to determine if *m*-tyrosine activity in soil is impacted by availability in these soil conditions. We also evaluated the relative soil persistence of *m*-tyrosine in different soil media over time. One key objective was to demonstrate that laboratory bioassays that evaluate allelochemical activity and soil persistence can be utilized to generate useful preliminary data, in an attempt to predict allelochemical activity under field conditions, and to design more appropriate field-based studies to evaluate allelochemical persistence in the rhizosphere environment.

Methods and Materials

General Procedures Soil (Arkport fine sandy loam: psamentic Hapludlafs, coarse loamy mixed mesic, pH 6.2) was collected from a field situated at the Bluegrass Lane Research Farm at Cornell University in Ithaca, NY. Soil was allowed to dry at room temperature and was sieved (1.8 mm sieve) and mixed with dried field or patio sand at a ratio of 1:1 to allow for enhanced permeability and reduced soil puddling in laboratory based experiments with small receptacles. The soil mixture had an organic matter content of less than 2.0%. In addition, a peat-based growth medium with higher organic matter (MetroMix 360, Scotts Company, Maryville, OH, USA, organic matter content >4%; pH 6.4) was utilized for experimentation to assess impact of high organic growth media upon *m*-tryosine activity (Roberts 2006). Soils were not autoclaved but were dried at room temperatures before utilization. DL *m*-Tyrosine was purchased from Sigma/Aldrich and was utilized in all experiments. In experiments performed to evaluate activity of synthetic *m*-tyrosine and *o, p*, and purified *m*-tyrosine, synthetic DL *m*-tyrosine showed nearly identical activity to that evaluated in purified root exudates of fine leaf fescue cv. Intrigue (Bertin et al. 2007). A stock solution was prepared (6 mg*m*-tyrosine/L) from which other concentrations of *m*-tyrosine dissolved in purified water were formulated for use in experiments outlined below. The pH of all *m*-tyrosine solutions and purified laboratory water (Waters Millipore Corporation, Billerica MA, USA) was within a neutral pH range (6.2–6.7) during the course of this experiment.

Soil Texture Study Soil sand media (40 g) was amended with 25 ml each of aqueous solution containing m-tyrosine in various concentrations to provide a final concentration in each Petri dish of 20, 40, 80, and 160 μM*m*-tyrosine per dish. Soil that was amended with 25 ml untreated purified water served as a control so that each dish was moistened with an equal volume of water before an experiment was conducted. All dishes were closed with lids after seeding with indicator species, and placed in an controlled environment incubator until experiment termination. A similar experiment was performed with MetroMix 360 growth medium (described above) utilizing an identical protocol.

Soil Amendment Studies MetroMix 360 (15 g) growth medium was amended with 0, 0.25, 0.5, and 1 g of activated carbon (Sigma Corporation, St Louis MO, USA) and with *m*-tyrosine solutions at a concentration of 160 µM. The experiment was replicated three times for each activated carbon treatment and was repeated over time.

An additional experiment was designed where MetroMix 360 (15 g) was amended with *m*-tyrosine to formulate concentrations of 20, 40, 80, 160 µM *m*-tyrosine in soil media. For each *m*-tyrosine concentration, 0, 0.25, 0.5, and 1 g/dish of activated charcoal treatments were evaluated. Soil that was amended only with distilled water or activated charcoal alone served as a control. Appropriate amounts of ammonium nitrate (NH_4NO_3) were dissolved in water and added to soil to provide final concentrations of 0.25, 0.5, 0.75, and 1.0 mM ammonium nitrate per dish. In addition, *m*-tyrosine was added to soil at a concentration of 160 µM.

Soil Persistence Study MetroMix media (15 g) was amended with 25 ml of appropriate aqueous solutions to provide 160 µM *m*-tyrosine per dish. Lettuce seeds (Johnny's Select Seed; Winslow, ME, USA) were sown at d 0, 2, 5, 7, and 15 d after the aqueous solution of *m*-tyrosine was mixed with the soil media. Lettuce radicle length was measured for each seedling 7 d after seeding, and percent inhibition of radicle growth was recorded.

Growth Analyses In all experiments described above, lettuce was selected as the assay species of choice because it exhibited rapid and uniform germination. Large crabgrass (*Digitaria sanguinalis*) also was assessed in the first two experiments because it is a common monocotyledonous weed in turf settings and exhibits reasonably uniform and fast germination. Prior experiments have shown that *m*-tyrosine is a potent inhibitor of root growth of both large crabgrass and lettuce (Bertin et al. 2003b). Soil or MetroMix 360, was placed in 9-cm petri dishes, and 15 lettuce or crabgrass seeds were sown on the soil surface of each dish following amendment with m-tyrosine, ammonium nitrate, or activated carbon. Root and shoot length of lettuce and large crabgrass were recorded after 7 d of growth in an incubator at a temperature of 25ºC and lighting of 46 µmol photon $m^{-2}s^{-1}$. Each treatment was replicated three times, and was arranged in a completely randomized design. All experiments were repeated over time.

Statistical Analyses Each data set was analyzed statistically by using the general linear model (GLM) procedure (SAS Inst. 1998) for completely randomized design experiments. Means were separated by LSD at the $a=0.05$ level. Standard errors were calculated for each treatment mean where appropriate.

Results and Discussion

Soils Amended with m-Tyrosine A sand/soil mix with low organic matter content amended with *m*-tyrosine significantly suppressed both the radicle and hypocotyl growth of lettuce, and increasing concentrations exhibited greater inhibition of growth, with radicle elongation especially impacted (Fig. 1). *m*-Tyrosine was dissolved easily in aqueous solutions, and was applied to soil that was then thoroughly mixed to ensure uniform distribution. Application was made in a manner similar to commercial herbicide formulations that are used in turf and landscape settings, and were evaluated in past laboratory experiments for comparative toxicity purposes. *m*-Tyrosine at these concentrations was approximately 2–4 times higher in concentration than comparable labeled field application rates of soil applied pendimethalin, which currently is used widely for commercial control of weeds in established turf (data not presented). Treated lettuce initially was able to germinate under the conditions provided, but further growth at higher concentrations of *m*-tyrosine up to 160 µM was significantly reduced, indicating that *m*-tyrosine was available in the soil solution at these concentrations in quantities sufficient to control seedling growth of lettuce during the evaluation period.

Compared with previous Petri-dish bioassays performed on filter paper, the concentration of *m*-tyrosine required for growth inhibition in soil media was higher. The concentration of *m*-tyrosine required for 50% inhibition of root growth of lettuce (I_{50}) in soil is approximately 30 µM, whereas in a soilless assay, the I_{50} is approximately 8 µM. For large crabgrass (data not presented), the I_{50} value in soil media was approximately 25 µM. It is likely that in these

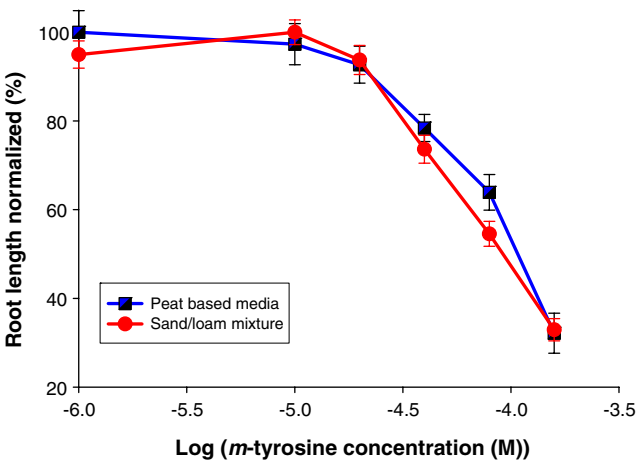

Fig. 1 Impact of *m*-tyrosine on root length of lettuce seedlings in an amended sand and loam soil mixture and MetroMix 360, a peat-based growth medium. Standard error bars are presented for each treatment and treatment means were calculated on the basis of 6 replicates per treatment and 90 seedlings per treatment unit

simulated soil experiments in the laboratory, *m*-tyrosine interacts by adsorption to soil particles or organic matter, resulting in reduced specific activity in soil or organic-based media because less is available in the soil water solution. Soil composition and texture often impact the activity of herbicides in a field environment, with higher concentrations of herbicide required in higher organic matter soils (Cobb and Kirkwood 2000). However, in the case of *m*-tyrosine, inhibition of lettuce radicle elongation was similar in both the higher organic matter growth medium and in the sand/soil mixture (Fig. 1). One might suspect that significant soil sorption, both reversible and irreversible, are associated with reduced activity or availability of *m*-tyrosine in soil media (Blum 1998) in comparison to toxicity observed in soilless growth media experiments performed on filter paper or agar media (Bertin et al. 2007).

Nitrogen Fertility Various levels of ammonium nitrate dissolved in water were added to soil amended with and without *m*-tyrosine. In the absence of *m*-tyrosine, nitrogen application did not significantly affect the growth of lettuce seedlings over a 7 day period, even at high concentrations of fertilizer N (1 mM). In the presence of *m*-tyrosine, it appeared that the addition of fertilizer N impacted root growth to some extent by reducing toxicity, especially at higher concentrations of soil N; however, toxicity was never overcome even at the highest N concentration (Table 1). Inhibition of radicle elongation was reduced by approximately 25% in the presence of *m*-tyrosine and 1 mM NH_4NO_3 in comparison to *m*-tyrosine applied without soil NH_4NO_3 (Table 1). We did not measure soil microbial activity in these assays, but higher microbial activity due to additional nitrogen fertilization might be suspected (Novak et al. 1995), thus resulting in significantly reduced availability of *m*-tyrosine due to enhanced microbial degradation. The presence or bioavailability of organic compounds as substrates for microbial growth generally influences microbial activity. However, additional experiments with various pasteurized or sterilized soil types and specific microbial communities will be useful to develop a better understanding of the role of soil microfauna on *m*-tyrosine toxicity, especially in field vs. laboratory conditions.

Table 1 Effect of *m*-tyrosine in peat based growth medium amended withsoluble ammonium nitrate solutions on root growth of 7 day old lettuce seedlings

Ammonium nitrate concentration (mM)	Root length of lettuce seedlings (cm) with *m*-tyrosine at 160 µM	Root length of untreated lettuce seedlings (SE=±1)
0	2.07 (0.02)	6.48 (0.37)
0.125	3.56 (0.07)	6.79 (0.32)
0.25	4.82 (0.1)	7.00 (0.29)
0.5	4.8 (0.09)	7.13 (0.35)
1.0	4.8 (0.12)	6.88 (0.47)

Numbers in parentheses refer to standard errors calculated for each treatment. Treatment means were calculated on the basis of 6 replications per treatment and 90 seedlings per treatment unit.

Soil Amended with Activated Carbon Activated carbon often is used to trap or eliminate the interference or inhibition caused by organic molecules in soil-based systems (Mahall and Callaway 1992; Inderjit and Foy 1999). By sequestering water-soluble organic constituents, the presence of activated carbon can remove allelochemicals from the soil in situ (Inderjit and Nilsen 2003). Activated carbon usually is used in field settings to remove residual herbicidal activity. Therefore, in this study, we assessed the activity of charcoal upon plant growth inhibition due to *m*-tyrosine. We compared the effect of *m*-tyrosine on lettuce seedling root elongation grown in soil bioassays supplemented with or without activated carbon. Compared to the control, soil amended with different amounts of charcoal clearly exhibited reduced toxicity of *m*-tyrosine on radicle elongation of lettuce (Figs. 2 and 3). Carbon itself had only limited effect on plant growth in untreated lettuce seedlings, and resulted in some apparent stimulation of growth that was not statistically significant. Inhibition of radicle elongation, however, was still observed at low concentrations of activated carbon (0–0.25 g/dish) in m-tyrosine treated seedlings. At the highest concentration of m-tyrosine and activated carbon (1.0 g/dish), inhibition

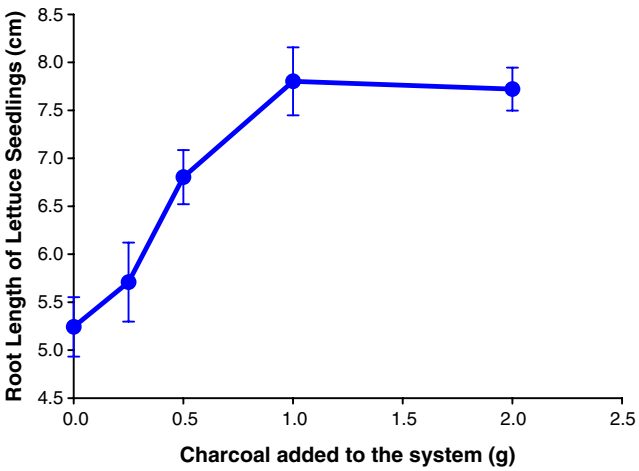

Fig. 2 Effect of increasing amount of activated carbon or charcoal upon root growth of *m*–tyrosine treated lettuce seedlings in peat based growth medium. M-tyrosine was applied at a rate of 160 µM to activated charcoal treated soils. Standard error bars are presented for each treatment mean and means were calculated on the basis of 6 replicates per treatment and 90 seedlings per treatment unit

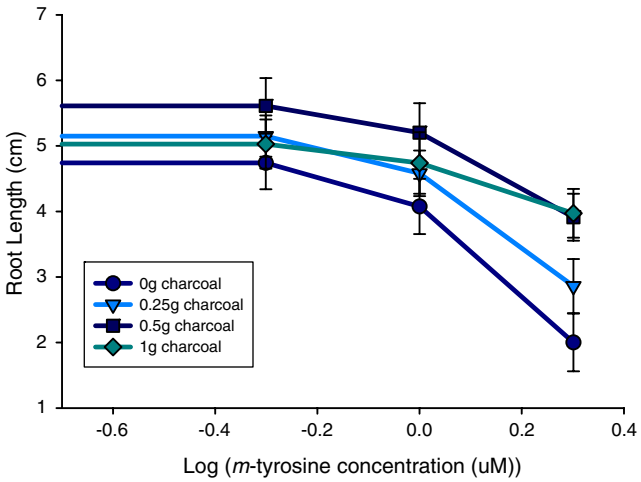

Fig. 3 Effect of increasing activated carbon or charcoal amendment and *m*-tyrosine concentration in peat based growth medium on root growth of lettuce seedlings. Standard error bars are presented for each treatment mean and means were calculated on the basis of 6 replicates per treatment and 90 seedlings per treatment unit

of root growth was much reduced in comparison to the m-tyrosine treatments without activated charcoal, suggesting that *m*- tyrosine was inactivated by high soil concentrations of activated carbon. This experiment suggests that *m*-tyrosine can bind to organic soil constituents such as carbon, thus resulting in reduced bioavailability and phytotoxicity, if these organic constituents are of fine texture and have available binding sites. In recent literature, it has been suggested that addition of activated carbon can cause increases in plant growth not because of reduced bioavailability of toxic constituents, but because of additional available nitrogen provided by the carbon itself (Lau et al. 2007). Although we did not assess available N from activated carbon in our growth media, we did show that additional N provided to the growth media in the form of ammonium nitrate impacted phytoxicity by causing reductions in toxicity. It may be that activated carbon may act in multiple ways to reduce toxicity of m-tyrosine, by reducing availability of the compound and also by providing additional soil N that might in turn stimulate microbial degradation. It is likely that significant levels of *m*-tyrosine remained available in the soil solution at high concentrations of m-tyrosine and were not completely sorbed even at higher carbon incorporation rates, given that we still observed considerable toxicity at these higher rates of incorporation (Fig. 3).

Soil Persistence Once allelochemicals are released into the environment, they are susceptible to decomposition or inactivation by microorganisms and also to chemical interactions that do not involve microorganisms (Rice 1984). Many environmental factors influence the persistence of allelochemicals in the soil (Blum 1998, 1999). A supplemental study was performed to evaluate the persistence of *m*-tyrosine in soil- based growth media over time (Fig. 4). When lettuce seeds were seeded in MetroMix 360 amended with *m*-tyrosine and resided at ambient temperature just prior to seeding, significant root and shoot growth inhibition were observed (Fig. 1). However, longer residence time in soil of 2 days or greater resulted in no significant soil toxicity in comparison to the untreated control. Our data indicate a half life of *m*-tryosine of less than 2 days and likely less than 24 hours after soil incorporation. Further experiments are required to determine the exact half life in these conditions. In the case of perennial species such as *Festuca* spp., consistent production and release of allelochemicals at phytotoxic levels is possible as the mass of living fibrous roots that exist within a perennial turf sod is large, and new root growth and continous root exudation is likely. Although rapid degradation of *m*-tyrosine is likely in both laboratory and also field settings, continuous release of root exudates over time by the extensive living root system of fine fescues may result in continual replenishment, especially in the rhizosphere where germinating weed seeds are likely to be located. In comparison to other phenolics, which are degraded within hours after application to soil settings, *m*-tyrosine appears to exhibit similar low levels of soil persistence (Blum et al. 1991).

In summary, we demonstrated that *m*-tyrosine is active in a number of soil settings when present in significant concentrations for uptake by seedlings from the soil solution. *m*-Tyrosine causes significant growth inhibition, in particular inhibition of radicle elongation of both

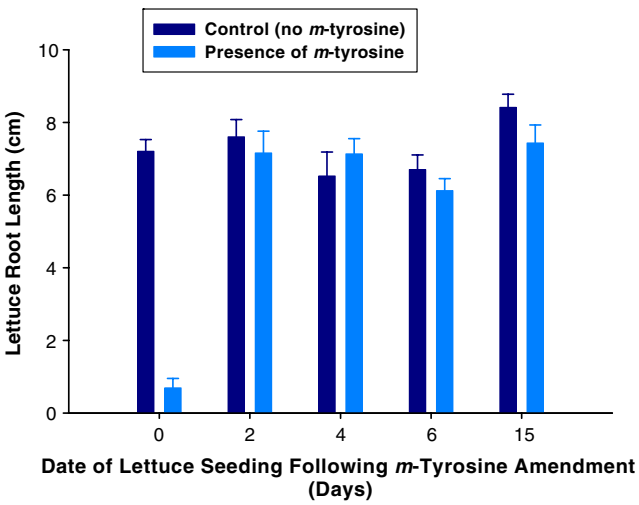

Fig. 4 Persistence of *m*-tyrosine in peat based growth medium as measured by lettuce seedling growth over time. *m*-Tyrosine was added to the soil media at time 0 and lettuce seeds were seeded after 0, 2, 4, 8, and 15 days following m-tyrosine amendment. Standard error bars are presented for each treatment mean and means were calculated on the basis of 6 replicates per treatment and 90 seedlings per treatment unit

monocots and dicots. Phytotoxicity is impacted by the addition of soluble nitrogen to the soil media, perhaps by stimulation of enhanced microbial degradation of *m*-tyrosine but apparently generally is unaffected by soil organic matter content or textural alteration. However, the toxicity of *m*-tyrosine was impacted by the presence of activated carbon in soil media, which likely results in decreased bioavailability and a subsequent reduction in phytotoxicity. It is also possible that additional N provided by presence of activated carbon also impacts availability and activity of *m*-tyrosine. In sand/loam soils, the persistence of *m*-tyrosine is limited to less than 48 hours or less, with an estimated half life of less than 24 hours.

While it is possible that *m*-tyrosine plays an important role in allelopathic interactions observed in fine fescue species, further experimentation in actual field settings is needed to assess accurately its rate of release and relative persistence in rhizosphere settings. *m*-Tyrosine may have potential to be utilized as a natural or organic herbicide if bioavailability in field soil can be significantly increased by reformulation to allow for enhanced soil persistence over time.

A recent paper by Kaur et al. 2009, suggests that microbial degradation of *m*-tyrosine is high, and half life of *m*-tyrosine was estimated as less than 24 hours in laboratory experimentation. The authors challenged the ability of *m*-tyrosine to act as an allelochemical inhibitor in microbially active soils. As their results also were conducted under conditions optimal for microbial growth in a laboratory setting, we believe that additional studies performed in a field setting with living root systems present and field microbial populations will better predict residence times of m-tyrosine in natural settings and determine whether continual production and release of a molecule such as m-tryosine will in certain cases and soils, lead to an allelopathic interaction. The common example of black walnut juglone indicates that degradation of this phenolic occurs in a matter of hours in laboratory persistence experiments. These findings, however, conflict with actual field observations where black walnut toxicity is clearly observed in infested soils in long term experiments. In the case of a perennial sod such as that formed by fine leaf fescues, the potential exists for nearly continual production during the growing season and a localized release of root exudates within the rhizosphere to germinating weed seeds. Additionally, we have observed weed suppression by certain fine leaf fescues over multiple locations and years and have determined that this suppression is cultivar dependent (Bertin et al. 2009). Further studies are now underway to directly assess m-tyrosine release rates and to evaluate toxicity within the rhizosphere of living fine fescue roots.

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the cultured broth of this strain was not successful. Instead, the co-culturing of *Microcystis* with the *Brevibacillus* sp. enhanced the production of two volatile compounds, β-cyclocitral (Fig. 1) (Jüttner 1984; Jüttner and Höflacher 1985) and 3-methyl-1-butanol (Wright et al. 1991). The former had a characteristic lytic activity (Ozaki et al. 2008). It was confirmed that these volatile compounds were derived from the cyanobacteria themselves. Further, β-ionone (Fig. 1), geosmin, and 2-methylisoborneol (2-MIB) derived from cyanobacteria and similar volatile compounds, i.e., terpenoids, produced by plants, also had lytic activity. Although the minimum inhibition concentration (MIC) values of the cyanobacterial metabolites were estimated to be higher generally than those of compounds from plants, their local concentrations around a cluster of cyanobacteria might reach a sufficient concentration to cause auto-lysis, because they are intrinsic metabolites (Ozaki et al. 2008).

Among these volatile compounds, β-cyclocitral caused a characteristic color change in the culture broth from green to blue during the lysis process (see Supplementary Material Fig. 1). In the past, a similar color change had been found several times in the natural environment and was recently observed in Lake Tsukui (see Supplementary Material Figs. 2, 3, 4). Because it was hypothesized that β-cyclocitral is critical for the regulation of cyanobacteria in a freshwater ecosystem, the blue color formation of cyanobacteria with β-cyclocitral was investigated. This study focuses on the lytic behavior of various cyanobacteria with β-cyclocitral and its mechanism involving the blue color formation.

Methods and Material

Chemicals β-Cyclocitral was purchased from Wako Pure Chemical Industries (Kyoto, Japan), β-ionone was obtained from Kanto Chemical (Tokyo, Japan), and citral, cinnamaldehyde, perillaldehyde, and vanillin were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Methanol was purchased from Nacalai Tesque (Kyoto, Japan).

Cyanobacteria Cultures Axenic strains NIES-73, -102, -103, -112 and -298 belonged to *Microcystis,* NIES-611 was a *Phormidium*, and NIES-808 was an *Anabaena*. They were

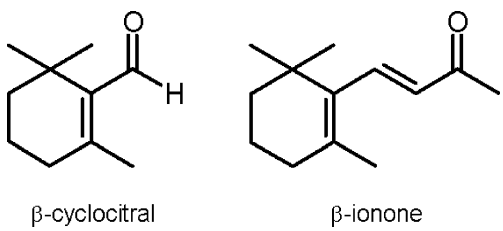

Fig. 1 Structures of β-cyclocitral and β-ionone

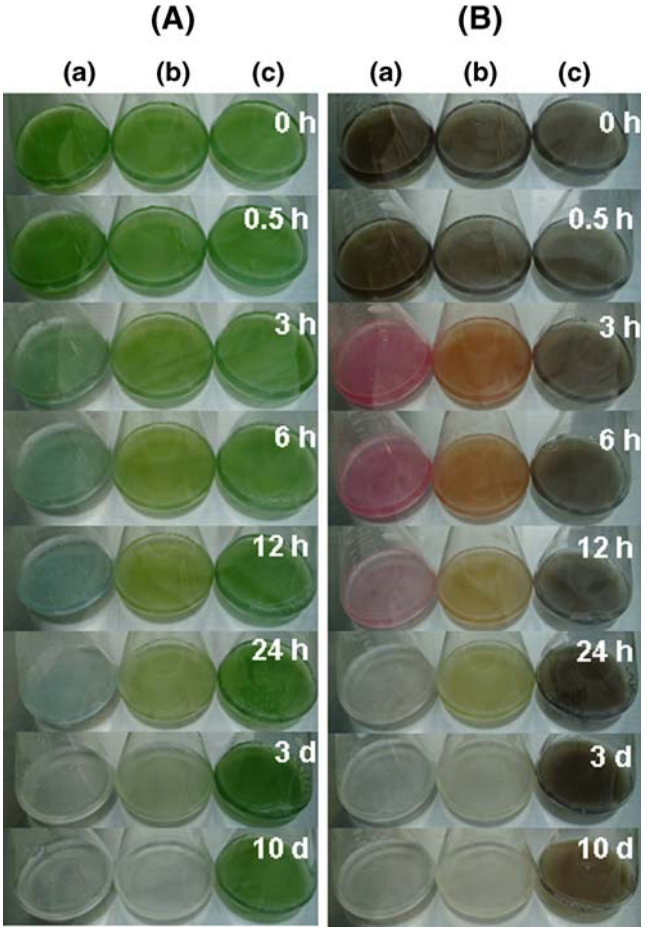

Fig. 2 The color change in lysed cyanobacteria; (A) NIES-102, (B) NIES-611, (a) β-cyclocitral, (b) β-ionone and (c) water

obtained from the National Institute for Environmental Studies (NIES), Tsukuba, Japan. PCC6803 was a *Synechocystis* sp. obtained from the Pasteur Culture Collection of Cyanobacteria (PCC), Paris, France. These strains were cultured in 1 liter Erlenmeyer flasks, each containing a modified MA medium (300 ml) at 25°C for 8 d under a 28 $\mu Em^{-2}s^{-1}$ continuous illumination. The MA medium consisted of a mixture of bicine (500 mg); $Ca(NO_3)_2{\cdot}4H_2O$ (50 mg); KNO_3 (100 mg); $NaNO_3$ (50 mg); Na_2SO_4 (40 mg); $MgCl_2{\cdot}6H_2O$ (50 mg); β-Na_2glycerophosphate (100 mg); a mixed metal solution (1 ml; composed of 1 mg of Na_2EDTA, 0.1 mg of $FeCl_3{\cdot}6H_2O$, 1 mg of $MnCl_3{\cdot}4H_2O$, 0.1 mg of $ZnCl_2$, 1 mg of $CoCl_2{\cdot}6H_2O$, 0.16 mg of $Na_2MoO_4{\cdot}2H_2O$ and 4 mg of H_3BO_3 in 200 ml of distilled water), and the resulting solution was adjusted to pH 8.6.

Collection of Cyanobacterial Blooms A bloom sample was collected from Lake Suwa on August 6, 2007, and another from Lake Tsukui on August 24, 2007 in Japan.

Measurement of Anticyanobacterial Activity The anticyanobacterial activity also was determined by measuring the

absorbance of chlorophyll-a (Uchida et al. 1998). Briefly, the final concentrations (6.5 mM) of the volatile compounds (β-cyclocitral, β-ionone, citral, cinnamaldehyde, perillaldehyde, and vanillin) were added to 100 ml of the cultured cyanobacteria (8 d) and natural blooms, and incubated at 25°C with shaking for 1 and 10 d under a 28 $\mu Em^{-2}s^{-1}$ continuous illumination. Water was used as the control. Five ml aliquots of the culture medium were withdrawn according to the color change during the first day, and then once per day until the 10th day. Simultaneously, the culture broth was photographed. The pH of the withdrawn samples was measured with a pH meter Navi F-52 (Horiba Ltd., Kyoto, Japan) and filtered through a GF/A filter (Whatman International, Ltd., Maidstone, England). The cyanobacteria on the filters were soaked in 2.5 ml of methanol, and the resulting mixture was stored at 4°C in the dark overnight. A 200 μl aliquot of the supernatant was placed on a 96-well microplate well (Kartell, Milan, Italy), and the optical density was measured at 665 nm with an MPR-A4iII microplate reader (TOSOH, Tokyo, Japan). The anticyanobacterial activity was determined as a decrease in absorbance (see Supplementary Material Fig. 6).

Acidification of MA medium by the Volatile Compounds β-Cyclocitral and β-ionone were added to 100 ml of the MA medium or water to the final concentrations (6.5 mM), and incubated at 25°C with shaking for 1 and 20 h under a 28 $\mu Em^{-2}s^{-1}$ continuous illumination. Water and ethanol were used as the negative control. Aliquots of 5 ml of the MA medium or water were withdrawn during the first, 0.5, 3, and 18 h. The pH of withdrawn samples was measured with a Navi F-52 pH meter.

Absorption Spectra of Culture Broths β-Cyclocitral and β-ionone were added to 100 ml of the *Microcystis* NIES-102 or *Phormidium* NIES-611 cultured for 8 d at the final concentrations (6.5 mM), and incubated at 25°C with shaking for 1 and 10 d under a 28 $\mu Em^{-2}s^{-1}$ continuous illumination. Water was used as the control. Aliquots of 5 ml of the culture medium were withdrawn according to the color change during the first day, and then once per day for 10 d. Absorption spectra were recorded at 800–190 nm using a Jasco V-560 UV/VIS spectrophotometer (Jasco Int. Co., Tokyo, Japan).

Isolation of an Acidic Compound β-Cyclocitral was added to water with shaking for 20 h, and the produced acidic compounds were collected by liquid-liquid separation with ether. The main compound was isolated by using a preparative HPLC whose system consisted of a pair of CCPS pumps, an SD-8022 degasser, a CO-8020 column oven, a UV-8020 detector, and a PX-8020 system controller (TOSOH, Tokyo, Japan). The sample was filtered by using an Ultrafree-MC membrane centrifuge filtration unit (hydrophilic PTFE, 0.2 mm, Millipore, Bedford, MA, U.S.A.) and loaded onto a Cosmosil $5C_{18}$-AR-300 column (250×10 mm ID, particle size 5 mm, TOSOH, Tokyo, Japan). The mobile phase was methanol/water containing 0.1%(v/v) formic acid. The methanol concentration was increased from 60 to 90% for 25 min in

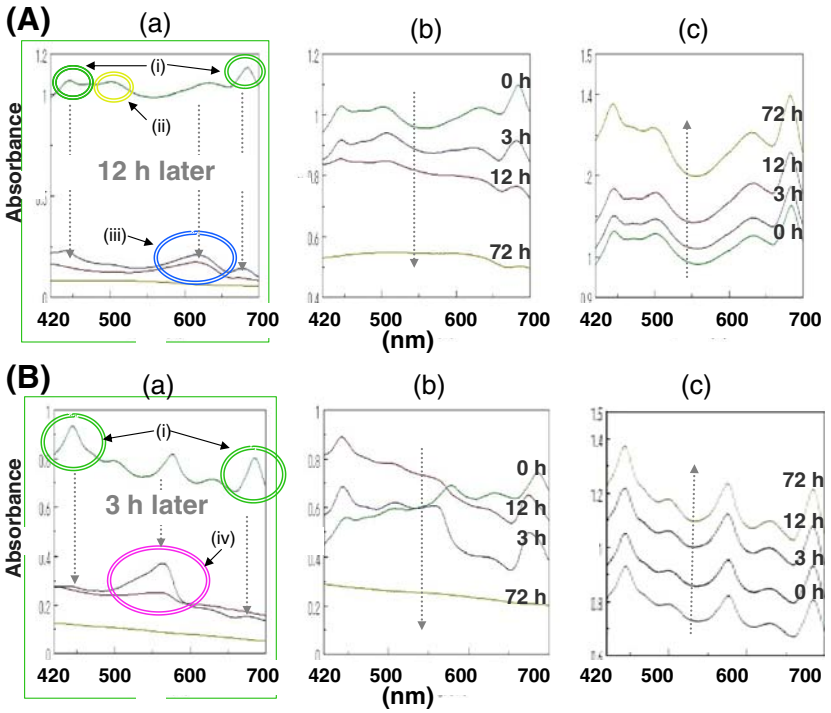

Fig. 3 The visible spectra of lysed NIES-102 and -611; (A) NIES-102, (B) NIES-611, (a) β-cyclocitral, (b) β-ionone, and (c) water. The circles of (i), (ii), (iii) and (iv) indicate the absorption maxima of chlorophyll-a (420 and 680 nm), β-carotene (500 nm), phycocyanin (620 nm) and phycoerythrin (560 nm), respectively. The absorbances of these characteristic absorption maxima changed with the growth of the cyanobacterium as indicated by the arrows

the linear gradient mode. The column temperature was 40°C and the flow rate was 3 ml/min.

LC/MS Five μl of the sample were filtered using an Ultrafree-MC membrane centrifuge filtration unit (hydrophilic PTFE, 0.2 mm, Millipore, Bedford, MA, U.S.A.). The acidic compound analysis was carried out by LC/MS. The LC separation was performed with an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, U.S.A.). The compound was monitored at 254 nm using a variable wavelength UV detector. The column was a TSK-gel Super-ODS column (100×2 mm ID, particle size 2 mm, TOSOH, Tokyo, Japan). The mobile phase was acetonitrile/water containing 0.1% formic acid. The acetonitrile concentration was increased from 10 to 80% for 20 min in the linear gradient mode. The column temperature was 40°C, and the flow rate was 0.2 ml/min. The MS analysis was accomplished with a Finnigan LCQ Deca XP plus mass spectrometer (Thermo Fisher Scientific, San Jose, CA, U.S.A.), equipped with an electrospray ionization (ESI) interface. The ESI conditions in the positive ion mode were as follows: capillary temperature, 350°C; sheath gas flow rate, 40 (arbitrary unit); ESI source voltage, 5000 V; capillary voltage, 46 V; tube lens offset, 30 V.

NMR Spectroscopy The isolated acidic compound was dissolved in chloroform-d_1. The ^{1}H- and ^{13}C-NMR spectra were measured by a JNM-ECA500 spectrometer (JEOL Tokyo, Japan) at 500.16 MHz and 125.77 MHz, respectively. The obtained signals are summarized as follows: $\delta^{1}H$ (ppm), 1.14 (6H, s); 1.44 (2H, m); 1.65 (2H, m); 1.77 (3H, s); 2.01 (2H, t). $\delta^{13}C$ (ppm), 18. 6; 21.6; 28.3; 31.7; 33.0; 38.6; 134.0; 136.5: 175.5.

Results and Discussion

Observation of the Blue Color Formation with β-Cyclocitral Figure 2(A) shows the effect of β-cyclocitral (a), β-ionone (b), and water (c) on the lysis of *Microcystis* NIES-102 after its addition. In contrast to the case with the water control (c), β-cyclocitral (a) and β-ionone (b) caused a lysis of the cyanobacteria as measured by a decrease in chlorophyll-a absorbance. While β-ionone gradually reduced the green color to provide a colorless solution mixed with white precipitates after 10 d, β-cyclocitral began to cause the characteristic color change from green to blue after 6 h, and the resulting blue color continued for about 1 d. The blue color change also was observed in *Microcystis* NIES-73, -103, -112, and -298, *Anabaena* NIES-808, and *Synechocystis* PCC6803 strains with β-cyclocitral. The *Phormidium* (NIES-611) strain originally provided a dark brown cultivated broth as shown in Fig. 2(B). Whereas the addition of β-ionone induced a gradual change from the original color to a colorless solution mixed with white precipitates via a diluted brown color [Fig. 2(B-b)], β-cyclocitral provided a characteristic pink color after 3 h, and this color continued for 12 h [Fig. 2(B-a)].

β-Cyclocitral and β-ionone were added to natural bloom samples from Lake Tsukui (see Supplementary Material Fig. 5). Generally, bloom samples of cyanobacteria have a sheath outside of the cells (Amemiya and Nakayama 1989; Amemiya et al. 1988) and we were interested in whether similar results were obtained when β-cyclocitral was applied to the bloom samples. Although the collected samples from Lake Tsukui were very dense, they were used without dilution. While the blue color formation was also observed after 4 d due to the addition of β-cyclocitral (see Supplementary Material Fig. 5), such behavior was not found in the case of β-ionone as stated above (see Supplementary Material Fig. 5). Therefore, β-ionone did not affect the chlorophyll-a concentration in the cells, and this behavior was different from that of the laboratory strains [Fig. 2(A-b) and (B-b)]. These results indicate that there was a difference between β-cyclocitral and β-ionone in the lytic activity against bloom samples composed of several cyanobacteria due to the presence of the sheath, and

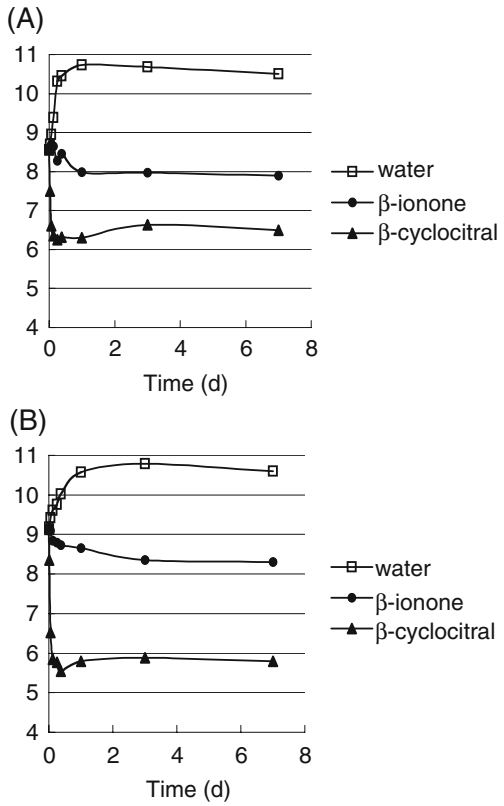

Fig. 4 Change in pH during lysis of cultured strain broths; (A) NIES-102, (B) NIES-611

that β-cyclocitral can lyse the laboratory strains of any genera and bloom samples, including many species of cyanobacteria, and can cause the characteristic color change from green to blue.

Visible Spectral Observation of Photosynthetic Pigments in Lysed Cyanobacteria In this experiment, we observed the visible spectra of the cultured broths affected by β-cyclocitral and β-ionone. Figure 3(A-c) shows the visible spectra of *Microcystis* NIES-102 with water as the negative control, and the absorbances of the characteristic absorption maxima increased with growth of cyanobacterium. These absorption maxima at 420 and 680 nm, 500 nm, and 620 nm can be assigned to those of chlorophyll-a, β-carotene, and phycocyanin, respectively. The addition of β-ionone uniformly reduced these absorption maxima to complete disappearance after 72 h [Fig. 3(A-b)]. In contrast, β-cyclocitral provided characteristic behavior in which the absorption maxima of chlorophyll-a and β-carotene disappeared, but that of phycocyanin remained after 12 h [Fig. 3(A-a)], indicating that the resulting blue color was derived from phycocyanin after the disappearance of the remaining pigments due to β-cyclocitral. This observation corresponded exactly to the phenomena shown in Fig. 2(A). An additional experiment using a *Phormidium* (NIES-611) strain, whose photosynthetic pigments are chrolophyll-a, β-carotene, and phycoerythrin instead of phycocyanin, which showed a pink color [Fig. 2(B-a)], supported the above conclusion [Fig. 3(B)]. That is, the addition of β-cyclocitral showed behavior similar to that of the absorption maxima disappearance of chrolophyll-a and β-carotene, but that of phycoerythrin at 560 nm still remained after 3 h (data not shown). This was consistent with the behavior shown in Fig. 2(B). These findings indicate that β-cyclocitral rapidly decomposed chlorophyll-a and β-carotene, so that the inherent colors from the tolerant water-soluble pigments became observable in the cultured broth.

Acidification of Cultured Broth and Bloom Sample with β-Cyclocitral The changes in pH of the cultivated broth of NIES-102 and -298 with the addition of β-cyclocitral and β-ionone are shown in Fig. 4. Thirty min after the addition of β-cyclocitral, the pH of both cultured broths decreased quickly to around 6, and it was maintained for several hours. However, such behavior was not observed in the case of β-ionone, in which the pH changed to around 8 over several hours. A dramatic pH change also was found in the bloom samples. As shown in Fig. 5, the pH decreased immediately to 4.5 after the addition of β-cyclocitral, and the pH then was slightly elevated for several hours in the case of the bloom sample from Lake Suwa. The bloom sample from Lake Tsukui showed nearly similar behavior. However, the pH changed to around 7 after the addition of β-ionone (Fig. 5).

We also investigated the pH-lowering effect of a volatile compound with an aldehyde group such as citral, cinnamaldehyde, perillaldehyde, and vanillin from plants, together with β-cyclocitral. Figure 6 shows the pH change due to the addition of these volatile compounds in water (A) and MA medium (B), in which water and ethanol were used as the negative control. The pH of a water solution of perillaldehyde gradually decreased to 4 in 20 h, and that of cinnamaldehyde decreased immediately to 4.5 in a manner similar to that of β-cyclocitral [Fig. 6(A)]. However, this behavior was not observed in the MA medium with buffer action [Fig. 6(B)]. In contrast, the solution decreased promptly to pH 4.5 after the addition of β-cyclocitral as already described.

Conversion of β-Cyclocitral to Corresponding Acidic Compound Twenty hours after the addition of β-cyclocitral to water, the reaction mixture was separated into the neutral and acidic fractions by liquid-liquid extraction. While a large amount of β-cyclocitral was still present in the former fraction, the acidic fraction contained several components as shown in Fig. 7. Because peaks No. 1 and No. 2 were not stable, it was impossible to isolate them. However, preparative HPLC could separate the main peak (No. 3) in a pure state, and the structural characterization was carried out as follows: the molecular weight

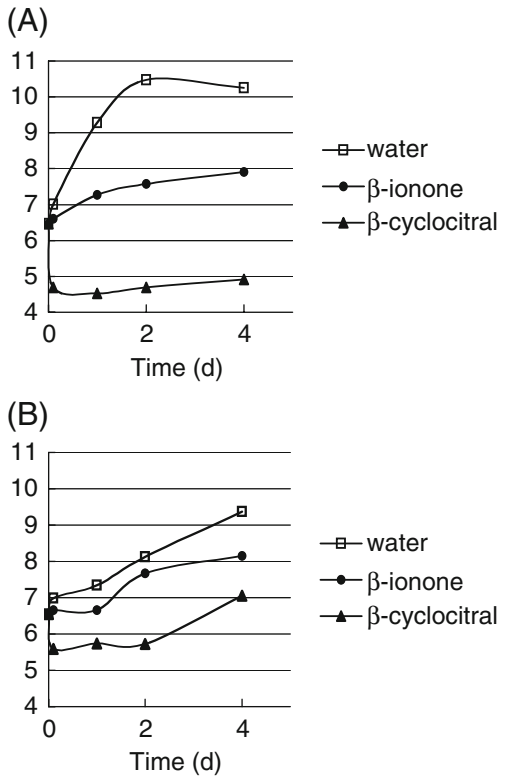

Fig. 5 Change in pH during lysis of bloom samples; (A) Lake Suwa and (B) Lake Tsukui

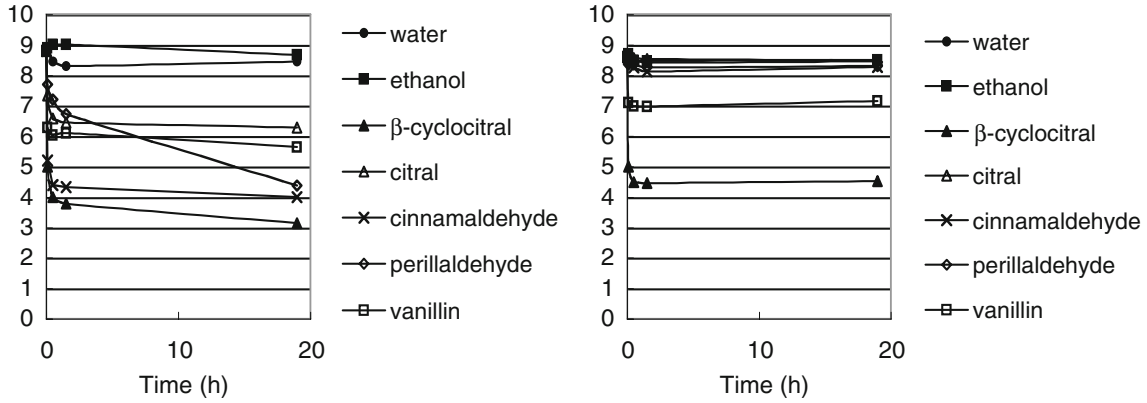

Fig. 6 Chang in pH with β-cyclocitral and terpenoids; (A) water and (B) MA medium

was determined to be 168 by LC/MS by using electrospray ionization (ESI), and the ^{1}H- and ^{13}C-NMR spectra were almost identical to those of β-cyclocitral except for an exchangeable proton, which was not observed in $CDCl_3$. From these data, we concluded that β-cyclocitral was converted to the corresponding carboxylic compound (2,6,6-trimethylcyclohexene-1- carboxylic acid) in a water solution. Similar results were obtained in MA medium. As mentioned above, β-cyclocitral showed the remarkable pH change in MA medium, but such behavior was not observed in the case of several other volatile compounds with an aldehyde group as shown in Fig. 6, thus suggesting that the pH-lowering effect was due to oxidation of an aldehyde group to a carboxylic acid. The solubility of the free acid was poor in water. One mg of the sample was not completely soluble in 1 ml of water in a short time period, and it took a few days to change to a clear solution, which resulted in a pH of about 3.3. Huang et al. (2002) reported that the blue color formation of a cyanobacterium (*Synechocystis* sp. PCC 6308) may be due to acid stress. They used hydrochloric acid for acidification, and the blue color was formed below pH 3.6. Probably, the acid formed from β-cyclocitral contributed to the pH-lowering effect to thus provide the characteristic blue color, although our resulting pH was higher than pH 3.6.

In summary, according to reports by Jüttner (1984), Jüttner and Höflacher (1985), and Watson (2003), β-cyclocitral is derived from β-carotene in *Microcystis* sp. Recently, it was found that β-carotene in cyanobacteria is cleaved by a specific enzyme (carotenoid cleavage dioxygenase, CCD) to provide retinal and related compounds (Kloer and Schulz 2006; Marasco et al. 2006). Probably, β-cyclocitral is formed together with 3-methyl-1-butanol by CCD in cyanobacteria and shows lytic activity against cyanobacteria. Because β-cyclocitral is more easily oxidized compared to similar aldehyde compounds, it is an interesting question whether β-cyclocitral or its acid contributes to the lytic activity. In a natural environment, cyanobacteria grow intensively during the summer season and gradually disappear in the autumn, and a sudden decline in the cyanobacteria has been observed in some cases (Fallon and Brock 1979). We observed a similar phenomenon together with blue color formation (Supplementary Material Figs. 2, 3, 4) in a lake. The present study provides support that β-cyclocitral plays an important role in this phenomenon. In a subsequent study, the dynamics of β-cyclocitral in cyanobacteria and the environment will be investigated in more detail.

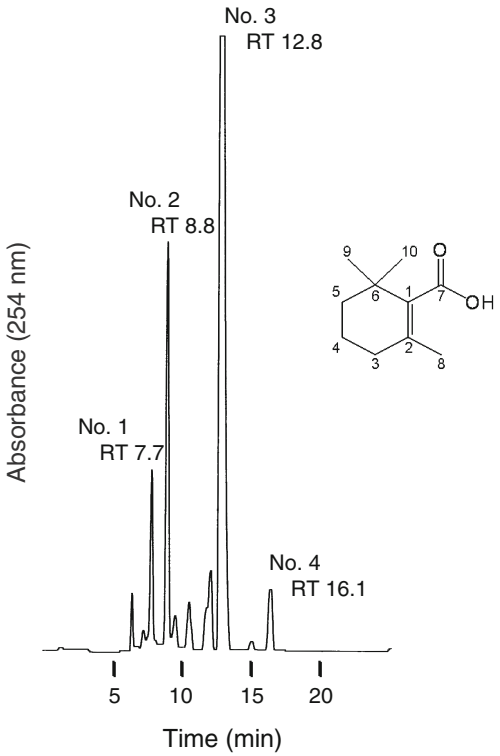

Fig. 7 HPLC separation of acidic compounds from the reaction of β-cyclocitral with water and the structure of the main compound (peak 3, 2,6,6-trimethylcyclohexene-1- carboxylic acid)

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tsugae, and *Fomes fomentarius*—each of which may be found within the same local area of forest.

Bolitotherus cornutus' primary defensive response is a death feint (Liles 1956), but, like many other tenebrionid beetles, it can produce volatile defensive chemical secretions (Tschinkel 1975a). The defensive secretions are stored in eversible glands at the tip of the abdomen (Tschinkel 1975b). When the beetle is disturbed, the glands are everted and stroked rapidly with the hindlegs, thus spreading the secretion (Conner et al. 1985). Although other species of tenebrionids secrete or spray defensive chemicals in response to attack by other insects (Peschke and Eisner 1987), *B. cornutus* reliably produces defensive secretions only in response to the warmth, humidity, and pulse—but not to the carbon dioxide content—of mammalian breath (Conner et al. 1985). Even mechanical injury by using artificial jaws does not usually result in eversion of the glands (Conner et al. 1985).

Early investigation of the chemical composition of *B. cornutus* defensive secretions was accomplished by directly "milking" the glands of the beetles and analyzing the product with packed-column gas chromatography (Tschinkel 1975a). With this older instrumentation, two large peaks were observed and identified as methyl-*p*-benzoquinone and ethyl-*p*-benzoquinone. Although other quinones and alkenes were detected in other tenebrionids, they were not detected in *B. cornutus* (Tschinkel 1975a). Solid phase microextraction, in combination with capillary gas chromatography-mass spectrometry (SPME-GC-MS) now provides an improved method for collecting, separating, and detecting volatile species. Recent applications of this technique to the detection of the quinone secretions of beetles include analysis of the defensive secretions of the red flour beetle, *Tribolium castaneum* (Herbst) (Villaverde et al. 2007) by using a carboxen/polydimethylsiloxane fiber, and analysis of the sex pheromones of a desert tenebrionid beetle by using a polydimethylsiloxane fiber (Geiselhardt et al. 2008).

Fieldwork on *B. cornutus* behavior suggested that beetles found on different species of fungus have slightly different scents to human observers. By using SPME-GC-MS, we compared the volatile secretions of male and female beetles collected from two species of fungus, *Ganoderma applanatum* and *Fomes fomentarius*, in the southern Appalachian Mountains.

Methods and Materials

Beetle Collection and Maintenance Forked fungus beetles were wild-caught as adults in the immediate vicinity of Mountain Lake Biology Station in Giles County, Virginia, USA in early August. Specimens were collected during the day from the surfaces of fungus brackets. They were placed immediately in containers that contained a fungal fruiting body harvested from that particular log, and shipped live to the laboratory for chemical analysis. Twelve individuals were collected from 6 colonies of *Ganoderma applanatum*, and 6 individuals from 6 colonies of *Fomes fomentarius* within an area of 0.05 ha. The sex of adults was determined easily by the large horns and ventral hair patches present only on males (Liles 1956). Analysis commenced 2 d after beetles arrived in the laboratory. The majority of beetles was analyzed within 1 wk of arrival; three beetles were not analyzed until over 1 wk later.

Chemicals Standards of *p*-benzoquinone (CAS 106-51-4), methyl-*p*-benzoquinone (CAS 553-97-9), 3-methylphenol (CAS 108-39-4), 3-ethylphenol (CAS 620-17-7), 2-methylhydroquinone (CAS 95-71-6), and 4-ethylresorcinol (CAS 2896-60-8) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Instrumentation A Varian (Walnut Creek, CA) Saturn 2100T gas chromatography—mass spectrometer (GC-MS), equipped with a FactorFour VF-5ms column (5% phenyl-methyl, 30 m, 0.25 mm i.d., 0.25-µm, Varian, Walnut Creek, CA, USA) was used for the analysis**.** Two cm dual-layered solid phase microextraction (SPME) fibers coated with highly cross-linked 50/30-µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) were purchased from Supelco (Bellefonte, PA, USA). Before use, fibers were cleaned by leaving them in the GC-MS injector port at 250°C for 30 min.

Sample Collection All sampling was done at room temperature by using headspace solid phase microextraction (HS-SPME). The amount of a compound collected by HS-SPME depends on both its gas-phase concentration and its affinity to the fiber (Zhang and Pawliszyn 1993); however, the maintenance of constant sampling conditions allows comparisons among samples. Before each run, a beetle was placed gently into a clean 10 ml glass vial and allowed to settle for several minutes. Then, by using a straw, human breath was blown onto the beetle for 5 sec. Gland eversion was observed in all cases. The vial was sealed immediately with a septum cap, and the SPME fiber was pierced through and exposed. After 3 min of collection, the fiber was removed and inserted into the GC/MS injection port, where it was desorbed for 30 sec at 250°C. In between runs, the SPME fiber was cleaned for 90 sec at 250°C to ensure that there was no carry-over from one beetle to the next.

A baseline measurement was performed each day by following the above procedure with an empty vial. Similarly, runs were performed by following the same protocol with a beetle present, but without exposure to breath.

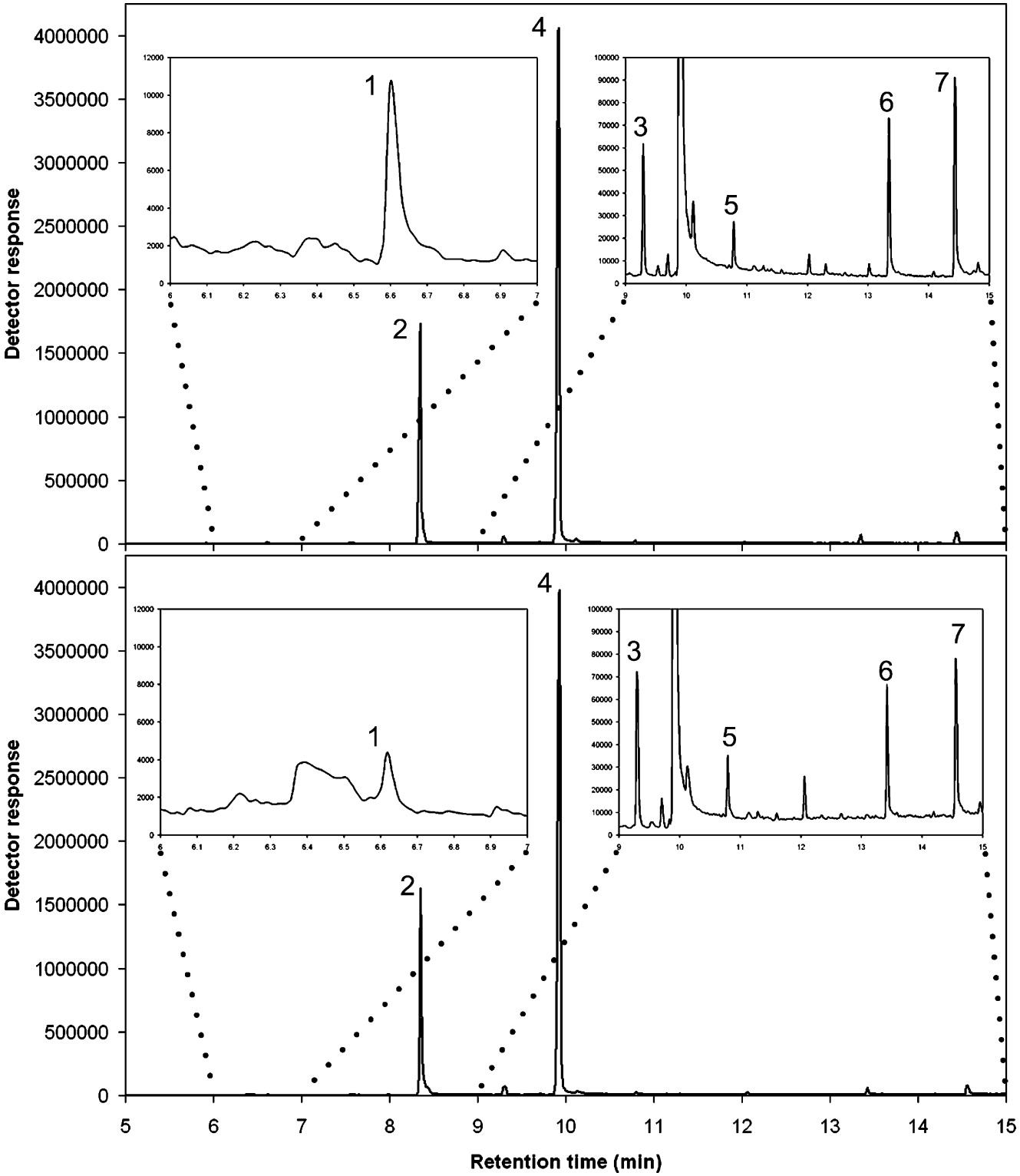


Fig. 1 Gas chromatogram of volatiles collected with solid phase microextraction from (*top*): a male *Bolitotherus cornutus* beetle collected from *Fomes fomentarius*; (*bottom*): a female *Bolitotherus cornutus* beetle collected from *Ganoderma applanatum.* 1: *p*-benzoquinone; 2: methyl-*p*-benzoquinone; 3: 3-methylphenol; 4: ethyl-*p*-benzoquinone; 5: 3-ethylphenol; 6: 2-methylhydroquinone; 7: 2-ethylhydroquinone. The scales for each portion of the graph are identical in the top and bottom pane

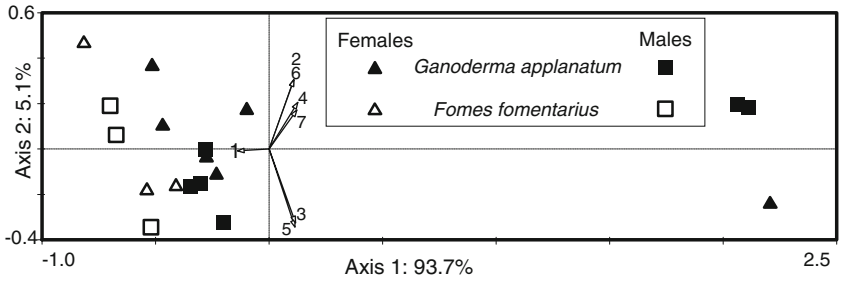


Fig. 2 Principal component analysis of centered log ratio transformed relative peak areas. 1: *p*-benzoquinone; 2: methyl-*p*-benzoquinone; 3: 3-methylphenol; 4: ethyl-*p*-benzoquinone; 5: 3-ethylphenol; 6: 2-methylhydroquinone; 7: 2-ethylhydroquinone

Gas chromatography-mass spectrometry A 20:1 split injection (column flow, He = 1.2 ml/min) into an injection port held at 250°C was used. Temperature programming: hold at 50°C for 3 min, ramp to 250°C at 10°C/min, and hold for 2 min.

With the exception of ethyl-*p*-benzoquinone and 2-ethylhydroquinone, all volatiles were identified by comparison of mass spectra and retention times with those of standards. Ethyl-*p*-benzoquinone (CAS 4754-26-1) was positively identified from its mass spectrum (NIST database), as well as by reference to prior identification of high concentrations of it in the defensive secretions of *B. cornutus* (Tschinkel 1975a). 2-ethylhydroquinone (CAS 2349-70-4) was identified tentatively by comparison of the mass spectrum to published data (Wahrendorf and Wink 2006), and by comparison of the retention time and mass spectrum to that of its structural isomer (with hydroxyl substituted at a different position on the ring), 4-ethylresorcinol.

Data Analysis As different beetles produced different absolute amounts of volatiles (statistically determined to be unconnected to sex or food source), a relative peak area was computed for each peak by dividing its area by the total area of the seven major, identified peaks for that beetle. Ordination analysis thus was done on fractional compositions that sum to unity, and for which the proportions of the different chemical compounds are not independent within a sample. Accordingly, a centered log ratio transformation (Aitchison 1984; Pawlosky-Glahn and Egozcue 2006) was employed prior to principal components analysis and its canonical equivalent, redundancy analysis, performed using CANOCO 4.5 (ter Braak and Smilauer 2002). In the case where a component was not detected, "0.00000001" was used in place of "0" as the relative peak area for that component in the calculation of centered log ratios (Aitchison and Egozcue 2005).

Results

The two major components of the beetles' volatile secretions were methyl-*p*-benzoquinone and ethyl-*p*-benzoquinone. However, smaller amounts of *p*-benzoquinone, 3-methylphenol, 3-ethylphenol, 2-methylhydroquinone, and 2-ethylhydroquinone also were detected (Fig. 1).

By using the chemical composition of the beetles' volatile defensive secretions, it is possible to differentiate beetles collected from *G. applanatum* from those collected from *F. fomentarius* (Fig. 2). The most important factor was the level of *p*-benzoquinone expressed. Three beetles collected from *G. applanatum* did not produce any detectable *p*-benzoquinone, and points for these beetles appear at the far right of axis 1 in Fig. 2. The points could, through their leverage, have a distorting or compressing effect on the rest of the ordination, thus inflating the effect of the *p*-benzoquinone. However, separation of beetles based on their fungal diet also was evident when the three points with high leverage were removed (Fig. 3). When fungal food source was used to constrain axis 1 of a redundancy analysis, the constrained axis explained 18.7% of the variance among beetles, and Monte Carlo permutations showed this axis to be significant (P=0.032). With the three beetles with no detectable *p*-benzoquinone excluded, the constrained axis was responsible for 23.8% of the

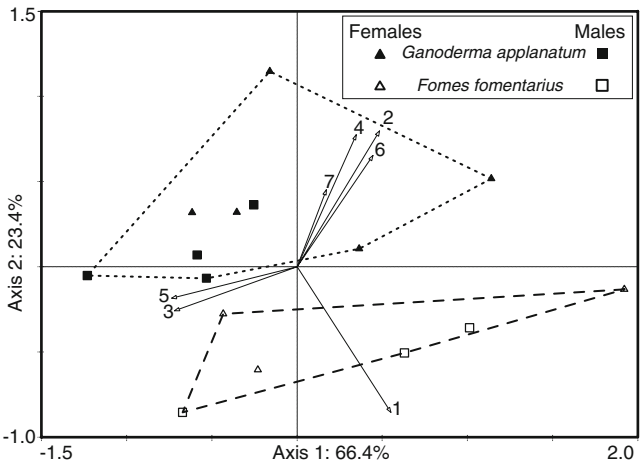


Fig. 3 Principal component analysis of centered log ratio transformed relative peak areas of defensive secretion of *Bolitotherus cornutus*, excluding three individuals collected from *Ganoderma applanatum* that produced no detectable *p*-benzoquinone. 1: *p*-benzoquinone; 2: methyl-*p*-benzoquinone; 3: 3-methylphenol; 4: ethyl-*p*-benzoquinone; 5: 3-ethylphenol; 6: 2-methylhydroquinone; 7: 2-ethylhydroquinone

Table 1 Chemical composition of volatile secretions of male and female forked fungus beetles collected from two species of fungus. Values are given as mean relative peak areas, expressed as a percent, with associated standard errors

Food		N	*p*-benzoquinone	Methyl-*p*-benzoquinone	3-methylphenol	Ethyl-*p*-benzoquinone	3-ethylphenol	2-methyl-hydroquinone	2-ethyl-hydroquinone
Ganoderma applanatum	Female	6	0.03±0.01	29±2	1.4±0.4	67±2	0.5±0.2	0.8±0.2	1.1±0.2
	Male	6	0.03±0.01	23±2	1.8±0.4	73±2	0.6±0.2	0.9±0.1	1.3±0.2
	All	12	0.033±0.007	26±2	1.6±0.3	70±2	0.5±0.1	0.9±0.1	1.2±0.1
Fomes fomentarius	Female	3	0.16±0.03	28±2	1.9±0.8	67±2	0.5±0.2	1.2±0.2	1.7±0.3
	Male	3	0.19±0.01	26±1	2±1	68.3±0.3	0.5±0.3	1.1±0.1	1.63±0.06
	All	6	0.18±0.02	27±1	1.9±0.6	68±1	0.5±0.2	1.2±0.1	1.7±0.1

variance among beetles, with $P=0.028$. Whether or not the three beetles with no *p*-benzoquinone were included, there was no evidence from the ordinations of differences in volatiles associated with beetle sex.

Although *p*-benzoquinone was a minor component of the detected volatile species, beetles that were collected from *F. fomentarius* produced, proportionally, a much greater amount than those that were collected from *G. applanatum* (Table 1). The lowest producing *F. fomentarius*-fed beetle still produced 1.4 times as much *p*-benzoquinone as the highest producing *G. applanatum*-fed beetle (as a proportion of the total volatiles detected for the beetle). The relative peak area for *p*-benzoquinone was significantly different for beetles collected from the two fungal food sources (Two sample *t* test: $t=8.94$, $d.f. = 16$, $P<0.001$).

Several significant relationships were observed among the components. As seen in Fig. 4, the relative peak areas of 3-methylphenol and 3-ethylphenol are positively linearly correlated ($r=0.95$, $N=18$, Bonferroni-corrected $P<0.001$), and methyl-*p*-benzoquinone and ethyl-*p*-benzoquinone are negatively linearly correlated ($r=-0.94$, $N=18$, $P<0.001$). 2-Methylhydroquinone and 2-ethylhydroquinone also have a positive association, although the trend is not as clear as in the other two examples ($r=0.71$, $N=18$, $P=0.02$). No significant correlation was observed between the phenols and the quinones, or between *p*-benzoquinone and any other compound.

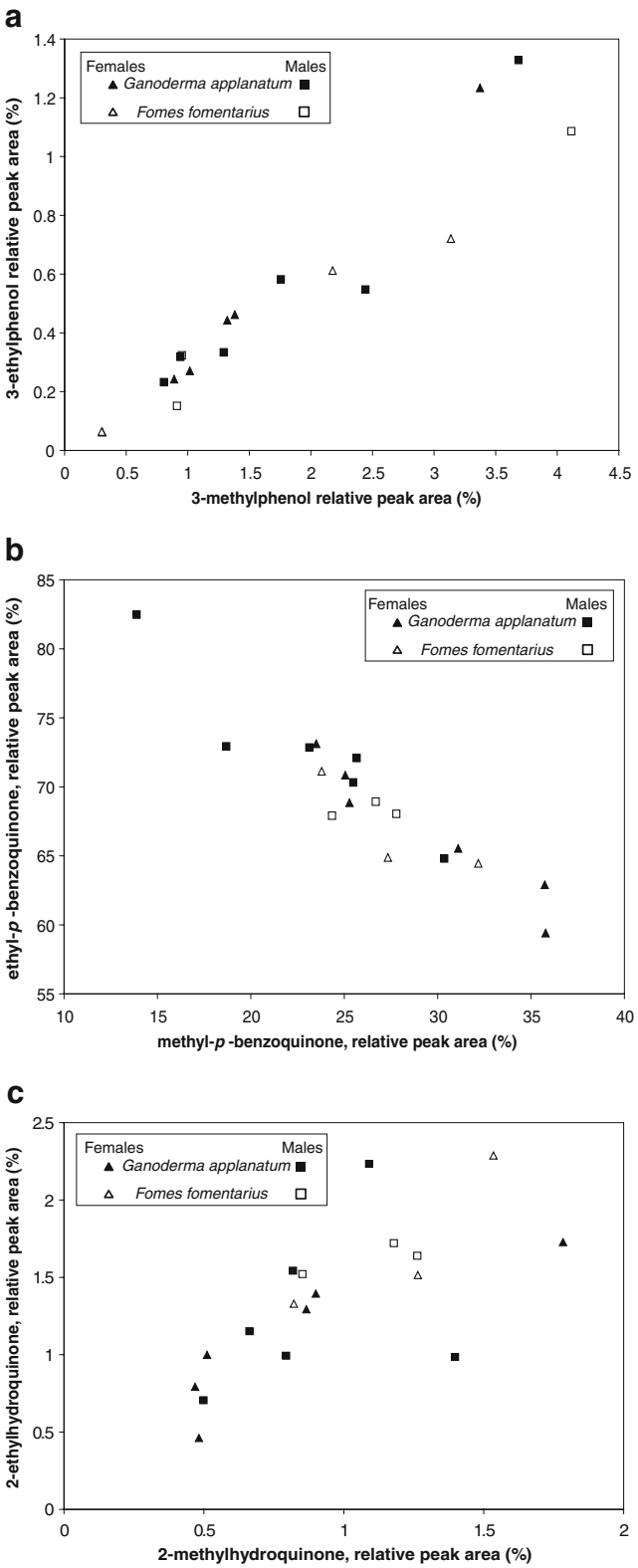
Discussion

Quinones are common in the defensive secretions of tenebrionid beetles (Tschinkel 1975a). Both methyl-*p*-benzoquinone and ethyl-*p*-benzoquinone previously were detected in the defensive secretions of *B. cornutus*, but the unalkylated *p*-benzoquinone was not (Tschinkel 1975a). Indeed, the lack of *p*-benzoquinone was seen as a distinguishing feature for *B. cornutus* among other tenebrionids that secrete quinones but not hydrocarbons (Tschinkel 1975a). We showed here that *p*-benzoquinone is produced, albeit in very small quantities, and that differences in diet and habitat may influence levels of this compound.

At first glance, it is surprising that *p*-benzoquinone has a much greater differentiating effect than similarly structured alkylated benzoquinones. However, this may be due to the presence of different biosynthetic pathways for the creation of alkylated benzoquinones (Meinwald et al. 1966). In *Eleodes longicollis* Lec., another tenebrionid beetle, Meinwald et al. (1966) demonstrated that *p*-benzoquinone was synthesized using the aromatic ring from either tyrosine or phenylalanine, whereas the alkylated benzoquinones were derived from acetate units. Therefore, differences in food sources may affect one pathway but not the other. *G. applanatum* fungus might have less bioavailable tyrosine or phenylalanine than *F. fomentarius*. Interestingly, some food source preferences have been observed in controlled observations of *B. cornutus* (Heatwole and Heatwole 1968), with *G. applanatum* being less preferred when another fungus species was available (*F. fomentarius* was not tested).

The three beetles collected from *G. applanatum* that did not produce any detectable *p*-benzoquinone were those that were sampled over a week later than the other beetles; perhaps some variation in living conditions caused the difference. However, these extremes of *p*-benzoquinone concentration did not enhance the separation between beetles collected from the two species of fungus, despite *p*-benzoquinone being the major distinguishing species between the two groups. In all other cases, a mixture of male and female beetles collected from *G. applanatum* and *F. fomentarius* were sampled on the same day.

3-Methylphenol (Tschinkel 1969; Attygalle et al. 1991; Villaverde et al. 2007; Geiselhardt et al. 2009;), 3-ethylphenol (Tschinkel 1969; Geiselhardt et al. 2009), 2-methylhydroquinone (Happ 1968; Wahrendorf and Wink 2006; Villaverde et al. 2007; Geiselhardt et al. 2009), and 2-ethylhydroquinone (Happ 1968; Hodges et al. 1996; Wahrendorf and Wink 2006; Geiselhardt et al. 2009) have all been detected in the defensive secretions of other tenebrionid beetles, although quinones are more ubiquitous



◀ **Fig. 4** Correlations between volatile components (relative peak areas, in percent) found for male and female *Bolitotherus cornutus* beetles collected from *Ganoderma applanatum* or *Fomes fomentarius*. **A** 3-ethylphenol as a function of 3-methylphenol. **B** ethyl-*p*-benzoquinone as a function of methyl-*p*-benzoquinone. **C** 2-ethylhydroquinone as a function of 2-methylhydroquinone

in tenebrionids. The presence of all of these chemical compounds emphasizes the likelihood that the alkylated benzoquinones found in *B. cornutus* are synthesized from acetate units.

3-Methylphenol, 2-methylhydroquinone, and methyl-*p*-benzoquinone are thought to be sequential members of a biosynthetic pathway that originates with 6-methylsalicylic acid (Gnanasunderam et al. 1984), itself formed from acetate (or malonate) units (Birch et al. 1955). Similarly, 3-ethylphenol, 2-ethylhydroquinone, and ethyl-*p*-benzoquinone are likely derived from 6-ethylsalicylic acid (Gnanasunderam et al. 1984), which differs in its synthesis by the use of a propionate unit in place of an acetate (or malonate) unit, as evidenced by isotope tracing experiments on ethyl-*p*-benzoquinone (Meinwald et al. 1966). We did not detect any salicylic acids in the defensive secretions of *B. cornutus*, but their methyl esters have been found in the secretions of another tenebrionid beetle (Gnanasunderam et al. 1984).

In view of this pathway, one way to explain the positive correlation between the relative peak areas of the phenols (or hydroquinones) is by attributing their relationship to a coupled series of biochemical reactions in which the formation of one leads to favorable conditions for the production of the other. The negative correlation observed between the relative peak areas of the alkylated quinones might be explained by a competitive inhibition of the oxidase used to form quinones from hydroquinones (Happ 1968). It is interesting that correlations were seen only between methyl and ethyl analogs at each structural level; no correlations were found between members of a biosynthetic pathway. The lack of correlation between either alkylated benzoquinone and *p*-benzoquinone further emphasizes its different biosynthetic origins.

No significant differences were seen between the sexes. Therefore, although both phenols (Geiselhardt et al. 2008) and quinones (Ruther et al. 2001; Geiselhardt et al. 2008) are used as sex pheromones in other beetle species, this does not appear to be the case for *B. cornutus*. This is not unexpected, as the volatiles are emitted in response to a specific threat stimulus.

The ecological effect of the observed differences in chemical blends is unclear. Although, in comparison to *p*-benzoquinone, the alkylated benzoquinones have been shown to have greater repellant effects against ants and induce greater topical irritation in cockroaches (Peschke and Eisner 1987). *Bolitotherus cornutus* emits these compounds only in response to detected threats from mammalian predators. Some human observers can detect qualitative odor differences in the blends, but do not notice differences in overall repugnance, although *p*-benzoquinone is known to be a powerful irritant (Eisner and Aneshansley

1999). Further experiments with isolated *p*-benzoquinone or blends of quinones that vary in relative ratios of *p*-benzoquinone will elucidate the importance of dietary differences to anti-predator function.

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deterrent properties of primary chemistry could evolve in response to selection from insect herbivory (Berenbaum 1995). Even in the absence of a direct functional role in deterrence, the ecological implications of primary nutrient and chemistry variation are significant. Numerous studies have demonstrated that primary nutrients can influence host plant selection by herbivorous insects, insect foraging behavior, growth and performance, the composition of insect communities, and possibly the evolution of host plant specialization in herbivorous insects (reviewed in Berenbaum 1995).

One especially significant primary nutrient for herbivorous insects is sterols. Sterols are triterpenoid steroid alcohols that are used by eukaryotic organisms to maintain cell membrane integrity, permeability, and fluidity. Furthermore, they are precursors for steroid hormones and are directly involved in developmental gene regulation (Behmer and Nes 2003). For most insects studied to date, cholesterol (Fig. 1) is the primary sterol incorporated into cell membranes and a necessary precursor to steroid hormones (e.g., ecdysteroids in arthropods). However, unlike many other organisms, insects are unable to synthesize the steroid nucleus *de novo* and must obtain sterols (i.e., cholesterol) either directly or indirectly from their diets (Clayton 1964). For example, carnivorous insects can absorb and directly utilize the cholesterol contained in their prey's tissues. For herbivorous insects, however, obtaining cholesterol poses a challenge. Plants synthesize hundreds of different sterol molecules (collectively known as phytosterols), but often

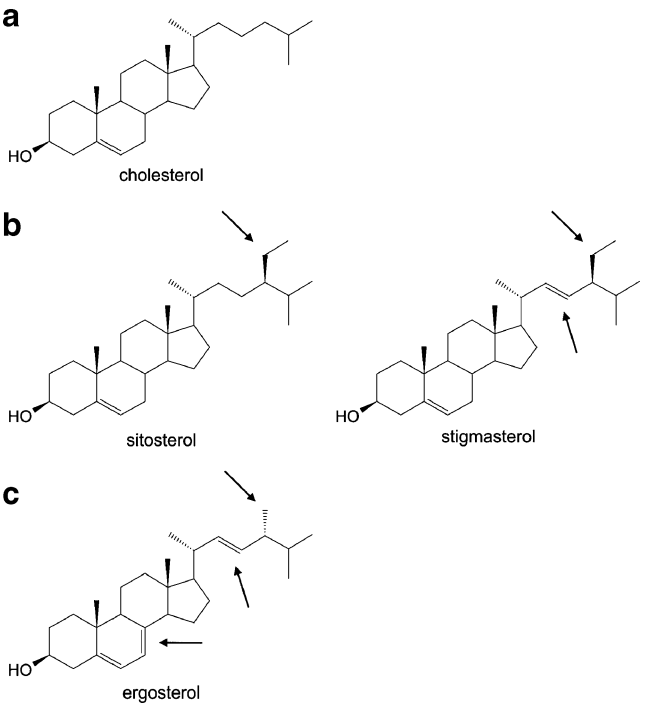

Fig. 1 The chemical structures of the most common (**a**) animal, (**b**) plant, and (**c**) fungal sterols. The black arrows highlight structural differences of plant and fungal sterols relative to cholesterol

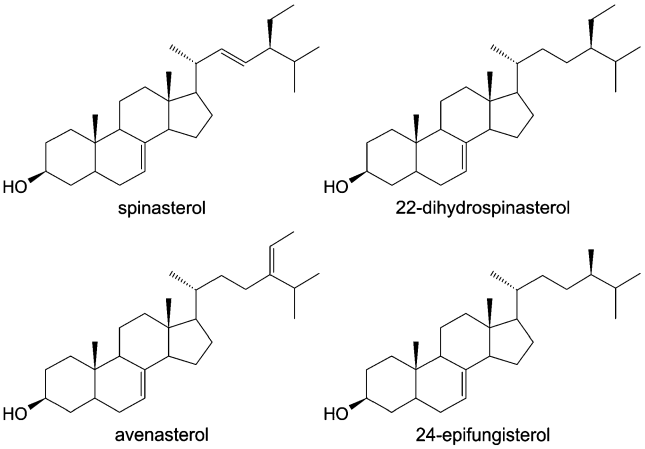

Fig. 2 The chemical structures of the sterols detected in the vegetative tissues of *Solidago altissima*

synthesize little or no cholesterol (Svoboda and Thompson 1985). Instead, the two most common phytosterols are sitosterol and stigmasterol (Fig. 1). Spinasterol also is prevalent, but relatively less common (Fig. 2). Phytosterols share gross structural similarities with cholesterol, but often have ethyl- or methyl groups at carbon position 24 and/or double bonds at positions other than five in the tetracyclic nucleus (B-ring) (Figs. 1, and 2). Consequently, in order to obtain cholesterol from their diet, most herbivorous insects must metabolize ingested phytosterols into cholesterol or other cholesterol-like molecules.

Two key factors complicate cholesterol acquisition for herbivorous insects. First, some species are unable to convert certain structural classes of phytosterols into cholesterol. Grasshoppers (Orthoptera: Acrididae), for example, are unable to convert sterols that contain Δ^7 and/or Δ^{22} double bonds into cholesterol (see Figs. 1, and 2 for examples) (Behmer and Elias 1999a; Behmer et al. 1999b). Like grasshoppers, a number of insects are unable to dealkylate the hydrocarbon side chain, and/or to remove double bonds on the side chain or within the tetracyclic nucleus (B-ring) of the sterol molecule (e.g., Svoboda et al. 1977, 1989; Kircher et al. 1984; Ritter 1984; Rees 1985; MacDonald et al. 1990). The inability of some insects to utilize and/or efficiently metabolize entire suites of phytosterols suggests that, in addition to their dietary necessity, the sterol physiology of plants could mediate a myriad of plant—insect interactions. Second, for reasons that are not entirely clear, plants frequently maintain a portion of their sterols in a conjugated form (Wojciechowski 1991; Moreau et al. 2002). Conjugated sterols are virtually identical to free sterols, except that they are covalently bonded at the 3β-OH group to either fatty acids (steryl esters) or carbohydrates (steryl glycosides or acylated steryl glycosides; Moreau et al. 2002). The relative amount of conjugated sterols varies among plant species, and even within species and individuals under

different environmental conditions, over time, or in different tissues (cf. Moreau et al. 2002). Conjugating phytosterols also may interfere with an insect's ability to absorb and/or metabolize those sterols, but relatively little is known about the ecological consequences of sterol conjugation.

Remarkably, despite decades of research on insect sterol metabolism, little is known about the ecology of plant-insect sterol physiology and metabolism. In particular, it is unknown how a community of herbivorous insects metabolizes the sterols found in their common host plant. Do communities of herbivores specializing on a host plant converge on a common sterol metabolic strategy? Or, does host plant variation along with variation in insect host breadth offer a variety of "sterol niches"? Are particular insect species constrained for their metabolism strategies and, if so, what patterns are evident? Here, we analyzed the sterol profile of the tall goldenrod, *Solidago altissima* L., and six of its common insect herbivores, including two gall-formers, and the fungal symbiont associated with one of the gall-formers. Specifically, we attempted to elucidate how insects from distinct taxonomic groups, with different phylogenetic histories, and with different ways of obtaining nutrients (feeding guilds) metabolize the phytosterols in their shared diet. We ask how much these insects vary in the metabolic translation of host plant phytosterols, and what the observed variation reveals about the contribution of plant primary nutrients to plant—insect ecology. We predicted that insects that were phylogenetically related would exhibit similar metabolic capabilities, but that insects could diverge in sterol metabolic strategies as they diverge more generally in dietary strategies.

Methods and Materials

Collection Sites All collections were made in August to October, 2007 and 2008, at a site in Tennessee and another one in Ohio, USA. The site in Tennessee was Shelby Bottoms Greenway and Nature Park in Davidson County (36.168°, −86.718°). This site is a protected 810 acre stretch of alluvial floodplain that runs parallel to the Cumberland River. It is characterized by large stands of open prairie and wetlands surrounded by light forest. The plant community throughout the open prairies is dominated by various grasses and *S. altissima*, but also includes other asteraceous species, such as *Ambrosia*, *Bidens*, *Cirsium, Eupatorium*, and *Rudbeckia*. Some of the common non-asteraceous genera include *Asclepias*, *Daucus*, *Desmodium*, *Rubus*, *Solanum*, and *Vitis*.

The second site was Beaver Creek Wildlife Area North in Greene County, Ohio (39.765°, −84.002°). Beaver Creek Wetland Wildlife Area North is a 380 acre portion of the protected Beaver Creek Wetland, specifically designated for hunting and fishing. This site includes sections of open prairie, marshland, and lightly wooded areas. The open prairie is dominated by various grasses and *S. altissima*, but also includes numerous other herbaceous asteraceous genera, including *Ambrosia*, *Aster*, *Bidens*, *Conyza*, *Erigeron*, *Eupatorium*, *Euthamia*, *Rudbeckia*, and *Symphyotrichum*. Some of the common non-asteraceous genera include *Asclepias*, *Daucus*, *Desmodium*, *Rubus*, *Solanum*, and *Vitis*.

Study Taxa This study focused on the plant *S. altissima*, a subset of insects observed on it, and a fungus found associated with one of the insects. A brief description of each taxon follows.

The Plant Solidago altissima (Asteraceae) is an herbaceous, rhizomatous perennial that inhabits old fields and disturbed habitats throughout a large portion of North America. Its range extends from southwestern Canada, south through the eastern Rocky Mountains to southeastern Texas, and east to the Atlantic seaboard and coastal plains of the Gulf of Mexico. *Solidago altissima* ramets emerge from a mother rhizome in late spring, grow throughout the summer, and flower from late August to early October. *Solidago altissima* prefers dryer, more circum-neutral soils, but is relatively tolerant of a wide variety of environmental conditions and is a dominant disturbed habitat species through a large portion of its range. *Solidago altissima* is attacked by over 100 species of herbivorous insects, including numerous non-specialists and a handful of *Solidago*/*Aster* specialists (Abrahamson and Weis 1997).

The Insects We focused on six locally abundant species that represented a range of feeding guilds, including a leaf-chewer, three phloem-feeders, and two gallers—one stem galler and one leaf galler. Our target insects also were taxonomically diverse, representing three orders and five families.

The leaf chewer was *Trirhabda virgata* LeConte (Coleoptera: Chrysomelidae). This beetle specializes on plants in the genus *Solidago,* including the species *S. canadensis* L., *S. altissima*, *S. gigantea* Ait., *S. rugosa* Mill., *S. juncea* Ait., and *S. missouriensis* Nutt. (Swigoňová and Kjer 2004). It also has been recorded feeding on *Euthamia graminifolia* (L.) Nutt. and *Aster* spp., although these plants are unlikely to be consumed regularly (Messina and Root 1980). This beetle has a broad geographic range that extends from southern Canada through the United States east of the Great Plains.

We collected three phloem-feeders. The first was *Acutalis tartarea* Say (Hemiptera: Auchenorrhyncha: Membracidae). This treehopper specializes on species in the Asteraceae, including several *Solidago* species, *Ambrosia* spp. (putative preferred host), *Helianthus* spp., *Bidens bipinnata* L., and *Callistephus chinensis* (L.) Nees (Tsai

and Kopp 1980). Records of it feeding on locust (*Robinia* spp.) also exist, but these records may be based on misidentifications (C. Dietrich, pers. comm.). It geographic range is broad, extending from southeastern Canada, throughout the eastern and southeastern US, and west to the Great Plains.

The other two phloem-feeders used were aphids in the genus *Uroleucon* (Hemiptera: Sternorrhyncha: Aphididae). The first of these, *U. luteolum* Willams, specializes on species in the Asteraceae, with *Solidago* spp. as its preferred hosts. It attacks several *Solidago* species and may attack species in the genera *Erigeron*, *Conyza*, and *Aster* (Blackman and Eastop 2006). Its range extends from southeastern Canada, throughout the eastern and southeastern USA, and west to Kansas and Nebraska.

The second aphid *U. nigrotuberculatum* Olive also appears to be an Asteraceae specialist, with *Solidago* spp. as its preferred hosts (Richards 1972). It also may occasionally attack *Zigadenus* spp. (Melanthiaceae) (Robinson 1985). Records from Japan, where it is invasive, indicate that it can feed on other members of the Asteraceae, including *Aster*, *Callistephus*, *Chrysanthemum*, and *Rudbeckia*, plus two other non-Asteraceae, *Zigadenus* spp. (Melanthiaceae) and *Oenothera erythrosepala* Borbás (Onagraceae) (Blackman and Eastop 2006). It is broadly distributed in North America, extending throughout much of southeastern Canada, the eastern USA into the midsouth, and as far west as Colorado.

Finally, two galling flies were collected. The first was *Eurosta solidaginis* subp. *solidaginis* (Diptera: Brachycera: Tephritidae), a stem-galling fly that specializes on only two *Solidago* species: *S. altissima* and *S. gigantea*. Rarely, *E. solidaginis* galls also have been recorded on *S. canadensis* and *S. rugosa* (Abrahamson and Weis 1997). *Eurosta solidaginis*' range extends from southeastern Canada throughout most of the eastern USA, which includes New England west to North Dakota, south through northern Texas, and throughout the southeast to northern Florida.

The second fly was the leaf-galling midge *Asteromyia carbonifera* (Diptera: Nematocera: Cecidomyiidae). It too specializes on the genus *Solidago*, but has been collected on dozens of different goldenrod species (Gagné 1968; J.O. Stireman, T.G. Carr, pers. comm.). Like a number of other gall midges in the family Cecidomyiidae, *A. carbonifera* maintains a symbiotic association with a fungus (see below) (Bissett and Borkent 1988), which females actively transport to oviposition sites in specialized structure called the mycangium (Bissett and Borkent 1988). Unlike many other plant galling insects, the galls of *A. carbonifera* are not composed of plant tissue, but rather are made up almost entirely of fungal mycelium. Many of the biological details of the *Asteromyia*-fungus symbiosis are not well characterized, including any nutritional relationships between *A. carbonifera* and the fungus (Gagné 1968; Bissett and Borkent 1988). Its range extends throughout southern Canada, south through the United States into Mexico, and from coast to coast in the United States, but it is most common in northeastern North America.

The Fungus Botryosphaeria dothidea (Moug.) Ces. & De Not. (Ascomycota: Botryosphaeriales: Botryosphaeriaceae) has been identified as the fungal symbiont of *Asteromyia carbonifera* (Bissett and Borkent 1988; E. M. Janson, J. O. Stireman, and P. Abbot, unpublished manuscript). It is a generalist, cosmopolitan plant pathogen and endophyte, that has been isolated from hundreds of plant genera in numerous families throughout the world (Farr et al. 1989; Smith et al. 1996). In addition to being associated with *A. carbonifera*, *Botryosphaeria* species/lineages appear to be associated with many cecidomyiids, and thus may represent a long evolutionary association with gall midges (Bissett and Borkent 1988).

Tissue Collection for Sterol Analysis Plant, insect and fungus were collected in the field and processed according to the following protocols. *Solidago altissima* leaves were hand removed directly from ramets, placed in sealable plastic bags, and transported in a chilled cooler. Leaves of all ages were picked, except those that had started to senesce. Leaves were immediately frozen whole at −80°C until analysis upon arrival at the laboratory.

The beetles, treehoppers, and aphids were collected directly from *S. altissima* plants in the field and killed by placing them into separate 50 ml plastic centrifuge tubes filled with 100% ethanol. Beetles and treehoppers were allowed to drop directly from the plants into the tubes, while aphids were removed with soft forceps. We collected insects without regard to their sex. For the beetles and treehoppers, only adults were available. For aphids, we collected both nymphs and adults. The specimens were stored at 4°C in 100% ethanol until analysis.

We collected *E. solidaginis* galls by clipping them from *S. altissima* stems and transporting them in a chilled cooler. Galls then were dissected, and larvae were killed and stored by placing them in a −80°C freezer until analysis. Entire leaves containing galls of *A. carbonifera* were collected directly from *Solidago* ramets and transported in a chilled cooler. Galls were dissected, and larvae/pupae were killed and stored by placing them in a −80°C freezer until analysis. No adult flies were analyzed.

In order to obtain enough tissue for analysis and to obtain uncontaminated sterol profiles for the fungal symbiont of *A. carbonifera*, sterol analysis was performed on mycelia from plated fungal cultures. *Asteromyia carbonifera* galls were excised from *S. altissima* leaves and surfaced sterilized with a sequential sterilization procedure

(10 sec in 95% ethanol, followed by 2 min in 10% bleach solution, and finally 2 min in 70% ethanol) and then placed on 2% malt extract agar until evidence of mycelial growth. Mycelia then were isolated using sterile technique and subcultured to obtain pure cultures. The identity of each fungal isolate analyzed was confirmed by phylogenetic reconstruction of the nrITS region (data not shown). Briefly, genomic DNA was extracted using the method of Arnold and Lutzoni (2007). The nrITS region was amplified in 20 μl PCR reactions (1x NEB standard PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.5 μM each primer, 1 M betaine, and 1 U NEB *Taq* DNA polymerase) using primers ITS4 and ITS5 (White et al. 1990) with a thermal cycler program that included 2 min at 96°C, followed by 30 cycles of 30 sec at 94°C, 45 sec at 52°C, and 45 sec at 72°C, finishing with a 10 min extension at 72°C. Amplicons were treated with SAP/EXOI to remove residual dNTPs and primers, and then sequenced at the Vanderbilt University DNA Sequencing Core Facility (Nashville, TN, USA) or the University of Arizona Genetics Core (Tucson, AZ, USA). Phylogenetic reconstruction was performed in MEGA 4.02 (Tamura et al. 2007). Reconstruction was performed using maximum parsimony with the close-neighbor-interchange (CNI) algorithm where initial trees were obtained by random addition of sequences (10 replicates). Clade support was estimated by1,000 bootstrap replications. The reconstruction included several *Botryosphaeria* spp. samples publically available on NCBI's GenBank, including sequences from the epitype specimen for *B. dothidea* (isolate CMW 8000). We employed a phylogenetic species concept and considered all isolates that belonged to the same monophyletic clade as the *B. dothidea* epitype sequence to be *B. dothidea* isolates. Only those isolates that met these criteria were used in sterol analysis.

Sterol Extraction and Analysis Samples used for insect sterol extraction and analysis were comprised of hundreds to thousands of individual collected in the same geographic location at the same time of year (although in two separate calendar years). The qualitative sterol profile of each study species did not vary significantly across space or time, so the reported sterol profiles are an average (numerous individuals, two geographic locations, different calendar years) sterol profile for the species in question. This approach was necessitated in part by the relatively small size of the study insects and the goal of the study, which was to characterize broad patterns of sterol metabolism in an insect herbivore community. The free-alcohol and conjugated sterol profiles for *S. altissima* were obtained from hundreds of leaves across numerous plants, and the fungal sterol profile was obtained from 20 different fungal isolates.

Fresh frozen plant material, fresh fungal material, or ethanol preserved insect carcasses were extracted by obliteration using 8, #5 glass beads (Sigma, St. Louis, MO, USA) and a modified paint shaker. The obliteration was repeated 3 times for each sample in a 50 ml centrifuge tube, with constant shaking for 30 min in 30 ml of 95% ethanol. Ethanol fractions were removed, combined, evaporated to dryness, and the residue was resuspended in 70% methanol:water. Steroids were extracted from the 70% methanol:water phase with water equilibrated hexane. The steroid fraction was evaporated to dryness under nitrogen and subsequently resuspended in a minimal volume of hexane for subsequent concentration and cleaning by thin layer chromatography (TLC) on Silica G (Alltech, Nicholasville, KY, USA—250 micron plate thickness) developed with toluene: ethyl acetate (9:1). The bands that co-migrated with sterol standards were scraped from the TLC plate and extracted from the silica in anhydrous ethyl ether. The ether fraction was evaporated to dryness under nitrogen, and the resulting film was resuspended in hexane. Sterols were concentrated by repeated injections and collection of sterol containing fractions from a reverse-phase liquid chromatography (RP-HPLC) column. This was accomplished by eight, 50 μl injections per sample on an Apollo C18 high efficiency column (Alltech, Nicholasville, KY, USA) and eluted with acetonitrile:MeOH (9:1, 1 ml/min) at 38°C.

Next, sterol fractions were collected, combined, and evaporated to dryness under nitrogen and resuspended in 50 μl of hexane for subsequent injection on a gas chromatograph—mass spectrometer (GC-MS) for conclusive identification. Steroids were identified and quantified by GC-MS using previously produced standard curves for all steroids. Authentic sterol standards were purchased commercially (Sigma, St. Louis, MO, USA) or in the case of the Δ^7 sterols, isolated and purified from spinach. Steroids were analyzed by GC-MS on a 6850 networked gas chromatograph (Technologies, Inc., Santa Clara, CA, USA) and a 5973 mass selective detector (Technologies, Inc., Santa Clara, CA, USA) by using the following conditions: Inlet temp 260°C, transfer line temp of 280°C, and column oven temperature programmed from 80 to 300°C with the initial temperature maintained for 1 min and the final one for 20 min (ramp rate: 30°C/min). The column used was a glass capillary MS-5 column (30 m) (Restek, Bellefonte, PA, USA) with a film thickness of 0.25 mm. Helium at a flow rate of 1.25 ml/min served as carrier gas. The Agilent 5973 mass selective detector maintained an ion source temperature of 250°C and a quadrupole temperature of 180°C. All steroids were in agreement with authentic sterol standards at each of the separation, concentration, and identification steps. We report (1) cholesterol, (2) spinasterol, (3) 22-dihydrospinasterol, (4) avenasterol, (5) ergosterol, (6) brassicasterol (ergosta-5, 22-dien-3β-ol), (7) ergosta-7,22-dien-3β-ol, (8) 7-dehydrodesmosterol (cholesta-5,7,24-trien-3β-ol, (9) desmosterol, (10) lathos-

terol, (11) stigmasterol, (12) sitosterol, and (13) 24-epifungisterol within insect, fungi, or higher plant tissues. The mass spectrum for each of the sterols was consistent with previous studies. The prominent mass ions for all sterols are as follows: cholesterol *m/z* 386 [98%], 371 [50%], 368 [60%], 301 [56], 275 [100%], and 255 [50%]; spinasterol: *m/z* 412 [35%], 397 [25%], 369 [30%], 271 [95%] and 255 [100%]; 22-dihydrospinasterol: *m/z* 414 [99%], 399 [75%], 273 [75%], and 255 [100%]; avenasterol: *m/z* 412 [25%], 397 [25%], 314 [90%], 271 [100%], and 255 [80%]; ergosterol: *m/z* 396 [60%], 363 [100%], 271 [30%], and 253 [60%]; brassicasterol: *m/z* 398 [100%], 383 [16%], 380 [20%], 271 [65%], and 255 [194%]; ergosta-7,22-dien-3-β-ol: *m/z* 398 [25%], 383 [15%], 355 [10%], 271 [100%], and 255 [30%]; 7-dehydrodesmosterol: *m/z* 382 [80%], 349 [100%], 323 [40%], 271 [10%], and 253 [30%]; lathosterol: *m/z* 386 [100%], 371 [29%], 368 [3%], 273 [20%], and 255 [80%]; stigmasterol: *m/z* 412 [60%], 397 [15%], 379 [10%], 271 [80%], and 255 [100%]; sitosterol: *m/z* 414 [90%], 399 [60%], 396 [40%], 273 [60%], and 255 [100%]; 24-epifungisterol: *m/z* 400 [50%], 385 [25%], 367 [10%], 273 [5%], and 255 [100%].

Throughout the paper, free steroids are those identifiable in the tissue without further processing following ethanol extraction, while steryl-ester pools were reported following a subsequent base or acid hydrolysis step. Base saponification included the treatment of ethanol extracted sterols with a 5% ethanolic KOH solution at 70°C for 12 h, while acid hydrolysis included the treatment of ethanol extracted sterols with 0.5% H_2SO_4 in 95% ethanol at 50°C for 12 h. Subsequently, the freed steroids were extracted with hexane, backwashed to neutrality, and processed as described above for free sterols. Internal standard (100 μg cholestane) was added to select samples to determine extraction efficiency of the sterol extraction and concentration procedure. All data are presented as relative percentages of the total sterol profile identified within the given organism because no organismal weights were recorded. Where there is no report of sterol conjugates, the limited starting mass of material prevented us from dividing the sample for conjugate analysis.

Results

Solidago Leaf Sterol Profile In nature, sterols can exist in either a free- or conjugated form, and in the majority of plants the dominant form is the free-form. In the leaves of *Solidago altissima*, however, approximately 85% of the total sterol profile was in the conjugated form (Table 1), with the remaining 15% in the free-alcohol form. In terms of the composition of the free sterol pool, spinasterol (C-29 $\Delta^{7,22}$-sterol) was by far the dominant sterol (94%) (Table 1; Fig. 2). Three other free sterols were present, but at a low proportion of the total pool (Table 1; Fig. 2). In terms of the conjugated sterol pool, it consisted entirely of spinasterol. In this pool, approx. 63% of the spinasterol was conjugated to fatty acids/acetate, with the remaining fraction being conjugated to carbohydrates.

Externally Feeding Insects' Sterol Profiles The sterol profiles of the four external feeders showed tremendous variation in tissue sterol profile (Table 2; Figs. 1, 2, and 3), even though they were all collected directly from *S. altissima* plants.

Almost 60% of the sterol profile of the chrysomelid beetle *Trirhabda virgata* consisted of unmetabolized *S. altissima* sterols, with spinasterol comprising almost 50% of the total tissue sterol profile. Lathosterol (C-27 Δ^7-sterol), a potential metabolite of any *S. altissima* sterol following dealkylation, made up 40% of this beetle's tissue sterol profile. Only a small amount of cholesterol (1%) was detected.

We also observed multiple sterols in the tissues of the membracid treehopper *Acutalis tartarea*. Cholesterol (C-27 Δ^5-sterol) was the most abundant sterol (40%), but three unmetabolized plant sterols—stigmasterol (C-29 $\Delta^{5,22}$-sterol), spinasterol, and 22-dihydrospinasterol (C-29 Δ^7-sterol)—also were observed, at 30, 20, and 10%, respectively.

Interestingly, the two aphids showed marked differences in sterol profile, even though they are congeners and were feeding on the same plant part. *Uroleucon luteolum* showed a high cholesterol profile (90%), with the remaining 10% being divided equally among lathosterol, sitosterol (C-29 Δ^5-sterol), and stigmasterol. In contrast, *U. nigrotuberculatum* had much lower cholesterol content (just over 16%),

Table 1 Identification and relative percentages of sterols detected in the leaf tissue of *Solidago altissima*

	Sterol type (as relative % of specified sterol pool)			
Sterol form	Spinasterol	22-dihydrospinasterol	Avenasterol	24-epifungisterol
Free-alcohol (15[a])	94	4	1	1
Conjugated (85[a])	100	–	–	–

[a] these numbers represent the relative percentages of the total sterol pool that were in the free-alcohol or conjugated form.

Table 2 Identification and relative percentages of sterols detected in the tissues of four externally feeding insect species collected on *Solidago altissima*

Sterol source	Sterol type (% total sterol pool)							
	(1)	(2*)	(3*)	(4*)	(5)	(6)	(7)	(8)
Trirhabda virgata (Coleoptera: Chrysomelidae)	**1**	49	5	5	40	–	–	–
Acutalis tartarea (Hemiptera: Membracidae)	**40**	20	10	–	–	–	30	–
Uroleucon luteolum (Hemiptera: Aphidae)	**90**	–	–	–	3.3	3.3	3.3	–
Uroleucon nigrotuberculatum (Hemiptera: Aphidae)	**16.6**	45	–	–	15	6.6	6.6	10

1) cholesterol, (2) spinasterol, (3) 22-dihydrospinasterol, (4) avenasterol, (5) lathosterol, (6) sitosterol, (7) stigmasterol, and (8) 22-dihydrobrassicasterol. Sterols with asterisks were found in *Solidago altissima* (see Table 1). Values may not sum precisely to 100% because of rounding.

and almost half of its tissue sterol profile was unmetabolized spinasterol. Four other sterols were observed in *U. nigrotuberculatum*. One was lathosterol (15%), which is likely generated by the aphid dealkylating spinasterol or other *S. altissima* sterols. The remaining three were plant sterols (sitosterol, stigmasterol, and 22-dihydrobrassicasterol), but none was observed to exceed 10%.

Fungus Sterol Profile The dominant sterol in *Botryosphaeria. dothidea* was ergosterol at 95%. The other recorded sterol (ergosta-7,22-dien-3β-ol (5%)) may be a stable component of *B. dothidea*'s sterol biosynthesis pathway (Table 3; Figs. 1, and 3).

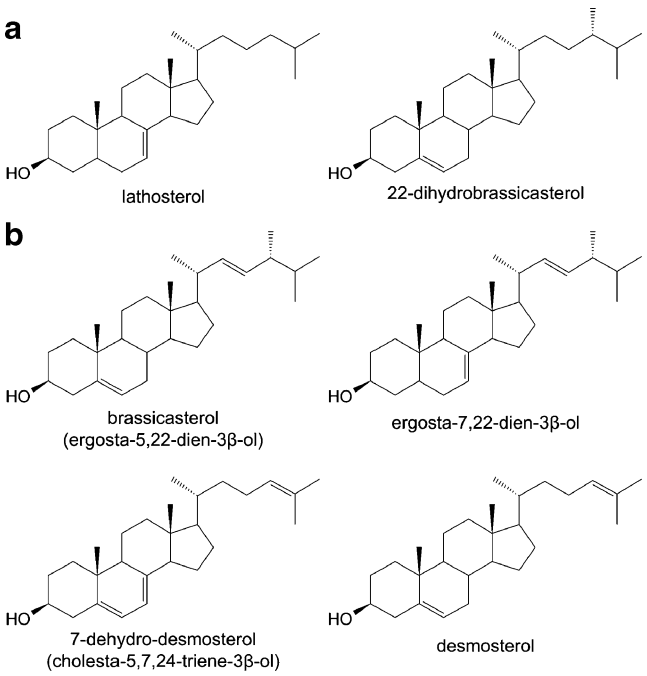

Fig. 3 The chemical structures of the additional sterols detected in the tissues of the insects associated with *Solidago altissima*. The two sterols shown in panel (**a**) are sterol products derived from phytosterols, while those shown in panel (**b**) are sterol products derived from fungal sterols

Internally Feeding Insects' Sterol Profiles The sterol profile of the leaf-galling midge *Asteromyia carbonifera* was completely free of any of the phytosterols detected in *S. altissima*. Instead, the profile contained ergosterol (C-28 $\Delta^{5,7,22}$) and ergosterol precursors/metabolites. This latter group of sterols included the C-27 ergosterol metabolite 7-dehydrodesmosterol (cholesta-5,7,24-trien-3β-ol), plus brassicasterol (ergosta-5,22-dien-3β-ol) and ergosta-7,22-dien-3β-ol (both are C-28 sterols). No detectable levels of cholesterol or 24-epifungisterol were found in *A. carbonifera*, although the common cholesterol precursor desmosterol (a C-27 $\Delta^{5,24}$-sterol) was found at low levels (3%).

In complete contrast to the leaf-galling *A. carbonifera*, the sterol profile of the stem-galling fly *Eurosta solidaginis* contained only phytosterols and phytosterol metabolites (Table 3; Figs. 2, 3). The majority (95%) of its tissue sterol profile was unmetabolized plant sterols, including spinasterol, 22-dihydrospinasterol, and avenasterol. The remaining 5% was mostly an unidentified sterol, plus a small amount of lathosterol. No 24-epifungisterol or cholesterol was detected in *E. solidaginis* tissue.

Discussion

Because insects must acquire sterols exogenously, their acquisition and metabolism may play important roles in insect ecology and community processes. Previous studies have demonstrated variation in sterol metabolism in insects, but have provided little ecological context. We characterized the sterol profiles of a natural insect herbivore community on the ecological model plant, the goldenrod *S. altissima*. Our data show that, despite sharing a host plant and a common sterol source, these insects exhibit marked variation in their sterol profiles, even between congeneric species. Part of this diversity likely is due to variation in host breadth and feeding guild among species. However, our data also highlight the likelihood of significant constraints on the evolution of sterol metabolism in

Table 3 Identification and relative percentages of sterols detected in two galling insect species and a fungal symbiont collected from *Solidago altissima*

	sterol type (% total sterol pool)								
sterol source	**(1)**	(2*)	(3*)	(4*)	(5)	(6)	(7)	(8)	(9)
Botryosphaeria dothidea (Ascomycota: Botryosphaeriaceae)	–	–	–	–	95	–	5	–	–
Asteromyia carbonifera (Diptera: Nematocera: Cecidomyiidae)	–	–	–	–	21	6	34	36	3
Eurosta solidaginis (Diptera: Brachycera: Tephritidae)	–	50	25	20	–	–	–	–	5

(1) cholesterol, (2) spinasterol, (3) 22-dihydrospinasterol, (4) avenasterol, (5) ergosterol, (6) brassicasterol (ergosta-5,22-dien-3β-ol), (7) ergosta-7,22-dien-3β-ol, and (8) 7-dehydrodesmosterol (cholesta-5,7,24-trien-3β-ol). Column 9 represents a consolidation of other sterols found in smaller amounts (≤ 5%). Sterols with asterisks were found in *Solidago altissima* (see table 1)

insects: most species, even *Solidago* specialists, were inefficient at metabolizing or were unable to use *S. altissima* sterols. Finally, our results underscore the importance of fungi as sterol sources for plant-associated insects, as witnessed in a number of other species (Kok et al. 1970; Mondy and Corio-Costet 2000; Noda and Koizumi 2003).

Compared to most species in the Asteraceae, which typically produce Δ^5-sterols (Nes and McKean 1977), *S. altissima* appears somewhat unusual in producing Δ^7-sterols as its principal sterols. However, this result is consistent with results from a previous pollen study of *Solidago* spp. where the principal sterol detected was 22-dihydrospinasterol (as Δ^7-stigmasten-3β-ol) (Svoboda et al. 1983). Here, it appears that specific subclades within the Asteraceae may contain plant species that synthesize primarily Δ^7-sterols, while other clades contain species that synthesize both Δ^7- and Δ^5-sterols, or Δ^5-sterols exclusively. For example, *S. altissima* belongs to the subfamily Asteroideae (Panero and Funk 2008), and in the Asteroideae sterol profiles from at least one species from seven different tribes have been recorded. The principal sterols in four of these (Anthemideae, Eupatorieae, Gnaphalieae, and Senecioneae) appear to be Δ^5-sterols. In contrast, two of the seven (the Calenduleae and Heliantheae alliance) contain species that synthesize both Δ^5- and Δ^7-sterols (Nes and McKean 1977). Finally, *Aster scaber*, which like *Solidago* belongs to the tribe Astereae, appears to synthesize Δ^7-sterols (spinasterol) exclusively (Tada et al. 1974). Thus, in clades that are basal to Astereae (e.g., Calenduleae), species that synthesize Δ^5:Δ^7-sterol mixes or only Δ^5-sterols may be common, while within the more derived Astereae, Δ^7-sterols (e.g., spinasterol) may be more common.

Many plants have independently converged on Δ^7 sterol profiles (e.g., Salt et al. 1991), yet the ecological and evolutionary significance of this convergence remains unclear. Δ^7-Sterols may confer important biochemical functions to the plant, and act in physiological adaptation to particular habitats (for example dry, saline environments; Salt et al. 1991; Behmer and Nes 2003). They also may act in defense against insects (Behmer and Nes 2003). Many insects cannot efficiently metabolize/utilize Δ^7-sterols, (Clark and Bloch 1959; Ritter and Nes 1981; Ritter 1984; Behmer and Elias 2000), and some insect herbivores learn to avoid foods that contain large amounts of unsuitable sterols (Behmer and Elias 1999b; Behmer et al. 1999a). Convergence could, therefore, be a result of (a) a history of intense selection from common insect herbivores that are unable to metabolize Δ^7-sterols (e.g., grasshoppers) and/or (b) biochemical adaptation to novel habitats and/or niches.

A surprising finding was that *S. altissima* contained mostly conjugated sterols. Most plants conjugate free sterols, but only a relatively small percentage of them (Wojciechowski 1991; Moreau et al. 2002). The function of conjugated sterols in plants and the ways insects metabolize them are unknown (Behmer and Nes 2003; Schaller 2003). Conjugation of plant sterols may be considered a form of plant defense. Possibly, conjugation may prevent some insects from accessing needed sterols since conjugated sterols may be "difficult-to-metabolize" forms. Further work is necessary to determine if conjugated sterols are an effective insect defense.

One of the most significant results is the remarkable variation observed in cholesterol content among insect herbivores. The virtual lack of cholesterol in the beetle (*T. virgata*) is somewhat surprising, but this is consistent with work done on the alfalfa weevil, *Hypera postica* Gyllenahl, which also specializes on plants (alfalfa) that contain only Δ^7-sterols (MacDonald et al. 1990). That a relatively large amount of lathosterol was recovered from *T. virgata* tissue demonstrates that side-chain dealkyalation likely occurs. Moreover, the fact that 99% of the tissue sterols recovered from *T. virgata* had a double bond at position seven (Δ^7) is consistent with the notion that many herbivorous insects lack the enzymes (specifically isomerases) necessary to completely convert Δ^7-sterols to Δ^5-sterols (Ritter 1984; Rees 1985; MacDonald et al. 1990). The detection of low levels of cholesterol raises the possibility that *T. virgata* can convert a small amount of Δ^7-sterol to cholesterol, or that an undetected Δ^5-sterol could have been dealkylated and converted to cholesterol. However, further work is necessary to differentiate metabolism from contamination (e.g., prior to

sampling, *T. virgata* may have ingested some material (e.g. pollen) that contained a small amount of cholesterol).

The high percentage of Δ^7-sterols in *T. virgata* suggests that Δ^5-sterols (like cholesterol) are not necessarily the primary sterol required for growth and development, as seen in some grasshopper (Behmer and Elias 2000) and leaf-cutter ant species (Ritter et al. 1982). For insects that regularly encounter Δ^7-sterols, such as *S. altissima* specialists, it may be more metabolically efficient to forego metabolism to cholesterol and instead use dealkylated Δ^7-sterols (e.g., lathosterol) and/or unmetabolized Δ^7-sterols for biological functions. However, for some generalist insects, the inability to metabolize Δ^7-sterols from host-plants has an adverse effect on growth and development, likely due to difficulties associated with ecdysteroid biosynthesis (Ritter and Nes 1981; Behmer and Elias 1999a, 2000). A puzzling issue remains for specialist insects that feed on plants with Δ^7-sterols and cannot introduce Δ^5 double bonds, in that most insect ecdysteroid biosynthesis pathways appear to require $\Delta^{5,7}$ sterol intermediates (Rees 1985), which these species would be unable to synthesize.

Given the significant amounts of cholesterol and lathosterol recovered in the two aphid species, our results indicate that aphids are capable of phytosterol dealkylation. However, the relatively small amount of cholesterol and lathosterol, and high amounts of spinasterol and other phytosterols suggests that, in *U. nigrotuberculatum,* dealkylation capabilities may be relatively inefficient. Similar results were observed in another aphid species (*Schizaphis gramimum* Rondani) fed Δ^5-sterols (Campbell and Nes 1983). The distinct difference in sterol profiles observed in the two *Uroleucon* species is notable, but the precise reasons behind the difference cannot be determined by this study. *Uroleucon* is a large genus that includes species that vary widely in their host-plant specificity and life histories. *Uroleucon luteolum* may simply be more efficient than *U. nigrotuberculatum* at converting phytosterols to cholesterol. One previous study hinted that phytosterol metabolism can show minor variability within a species (Behmer and Grebenok 1998). Thus, it is even more likely that substantial metabolic variation can exist between congeneric species.

Both *Uroleucon* species also contained detectable, albeit relatively low amounts of the Δ^5-sterols sitosterol and stigmasterol, neither of which was detected in *S. altissima* leaf tissue. Two possibilities may explain this result. First, recent evidence has shown that phloem phytosterol profiles do not always identically match that of the leaf tissue (Spencer T. Behmer, Robert J. Grebenok, and Angela E. Douglas, unpublished data), and *Solidago* may synthesize some Δ^5-sterols that are more highly concentrated in the phloem (see also Svoboda et al. 1983). Sap-feeders like aphids may be particularly likely to sequester 'whole plant' nutrients, vitamins, and sterols not immediately present at the feeding site. The second explanation is that aphids collected for analysis may have been feeding recently on plants that contained Δ^5-sterols, stored those sterols in a conjugated (esterified) form for later metabolism, and passed a fraction of those on to their offspring. Approximately 20% of the individuals in aphid samples were winged alates, which could have been migrants from other host plant species.

Interestingly, similar patterns were observed in the treehopper *A. tartarea*. *Acutalis tartarea* adults are phloem-feeders and highly mobile, so it is possible that the stigmasterol detected in their tissues was either transported by the *S. altissima* vascular tissues or obtained from a non-*S. altissima* plant species. A previous study of a planthopper (*Laodelphax striatellus* Fallen) suggests that some Auchenorrhyncha can dealkylate sterols (Noda and Koizumi 2003). It appears that *A. tartarea* may be inefficient at or incapable of metabolizing sterols with Δ^7 and/or Δ^{22} double bonds, given the lack of lathosterol and detectable levels of stigmasterol, spinasterol, and 22-dihydrospinasterol detected in its tissues. Further work, including direct sterol analysis of phloem itself and manipulative field experiments on confined insects, will provide insights into the unusual sterol variability in the phloem-feeders.

The sterol profiles of the two dipteran gall-formers were dramatically different. *Eurosta solidaginis* belongs to the family Tephrititdae, a large family of primarily herbivorous brachyceran flies. In addition to unmetabolized plant sterols, only trace levels of C-27 sterol were detected in larval tissues, suggesting that *E. solidaginis* is unable to dealkylate phytosterols. All brachyceran species studied to date are incapable of dealkylating C-28/C-29 sterols (Robbins 1963; Kircher et al. 1984; Svoboda et al. 1989). Lack of dealkylation is somewhat surprising, because species in herbivorous brachyceran families frequently may encounter low to non-existent levels of C-27 sterols in their plant-based diet. Moreover, fly larvae, especially the galling or leaf mining species common to the Brachycera, are relatively immobile, preventing them from incorporating behavioral mechanisms such as dietary mixing to cope with sterol inadequacies (e.g., Behmer and Elias 2000). Trace amounts of lathosterol in *E. solidaginis* may be transient components of the ecdysteroid biosynthesis pathway of *S. altissima* that were concentrated to detectable levels in *E. solidaginis* tissue. Other Brachycera (i.e., *Musca* and *Drosophila*) selectively absorb minute amounts of C-27 sterols from their diets for use in ecdysteroid production, as long as other sterols are present to act in structural roles (a phenomenon known as sterol sparing; Clark and Bloch 1959; Robbins 1963; Kircher et al. 1984; Feldhaufer et al. 1995). The capacity to successfully use C-28/29 phytosterols as membrane inserts may have been crucial for the shift that some brachyceran species have made from basal feeding modes such as

saprophagy/predation/parasitism/blood feeding (where C-27 sterols are abundant) to more derived herbivory (where C-27 sterols are relatively rare).

By contrast, *A. carbonifera*, a member of the midge family Cecidomyiidae, lacked plant-derived sterols altogether suggesting that it does not consume *S. altissima* tissue at all. Instead, it appears to acquire its sterols by consuming its fungal ectosymbiont. The presence of the C-27 ergosterol metabolite 7-dehydrodesmosterol (cholesta-5,7,24-trien-3β-ol) and the C-27 sterol desmosterol indicates that, like other Nematocera, *A. carbonifera* can dealkylate sterol side-chains (Svoboda et al. 1982). The dependency of *A. carbonifera* on its nutritional mutualist for dietary sterols adds to growing evidence that fungal mutualists can act as sources of dietary nutrients for plant-associated insects (Kok et al. 1970; Mondy and Corio-Costet 2000; Noda and Koizumi 2003). Indeed, many other cecidomyiids consume fungus as their principal food source (Bissett and Borkent 1988). Because *A. carbonifera* actively transports a single species of fungus for consumption (Bissett and Borkent 1988; E.M. Janson, unpublished manuscript), this interaction demonstrates how fungal symbioses could potentially circumvent some level of "nutritional mismatching" between an insect and its host plant (Janson et al. 2008). Here, the phytosterol metabolic capacity of *A. carbonifera* is irrelevant, because it can simply metabolize the sterols provided by its symbiont on all plants that it is able to gall.

The physiology and biochemistry of plant and insect sterol synthesis and metabolism has been characterized in great detail over the past 50 years. What has been largely ignored is how plant sterol physiology and the limitations of insect sterol metabolism affect the ecology of plant-insect interactions. What are the proximate and ultimate causes for the marked variation we observe in phytosterol physiology and insect sterol metabolism? Are phytosterols important in structuring insect communities? Do sterols shape how insects compete on their host plants or act as chemical defenses, and, if so, how often and under what ecological and evolutionary circumstances? To address such questions, a deeper understanding of the functional significance of the variation in phytosterol profiles among many plant species is necessary (e.g., Omoloye and Vidal 2007). These questions might best be addressed by correlating environmental conditions with particular phytosterol profiles across numerous phylogenetically independent plant groups, and determining the sterol metabolic capabilities of insect herbivores that co-occur with these plants. Such an approach would provide clues as to how important phytosterols act as defense against herbivores. Trade-offs associated with sterol specialization may affect host plant specificity. Furthermore, it is an open question as to how frequently and at what rate sterol metabolic capabilities are gained or lost. Insect taxa such as beetles, which have so far shown extreme variation in side-chain dealkylation, but consistency in B-ring metabolism (they are unable to introduce Δ^5 double bonds), may be especially amenable to such studies.

In summary, our results show that: 1) Several insect species were unable to fully metabolize *S. altissima* sterols into cholesterol, despite many of them specializing or feeding regularly on *Solidago*. Thus, there appears to be significant constraint on the evolution of sterol metabolism in insects in that many species appeared to be unable to convert or are inefficient at converting phytosterols with Δ^7 double bonds to Δ^5 sterols. 2) Closely related species and those occupying the same feeding guild differed dramatically in sterol metabolism. 3) Fungal symbionts can provide a source of dietary sterols for their insect hosts.

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and unsaturated hydrocarbons (Schmitt 1990; Oldham et al. 1994), mostly the same odd-numbered C21 to C31 ones (Schmitt 1990; Goulson et al. 2000; Saleh et al. 2007), suggesting that lipids from diverse body parts mix on the cuticle surface (Oldham et al. 1994). While probing for nectar, foraging bees touch flower corollae with various body parts, including the tarsi, and, thus, traces of cuticular lipids may pass onto the flower surface. Eltz (2006) speculated that the epicuticular wax of flower corollae could retain a record of past bumblebee visits. In a controlled garden experiment, flowers of the deadnettle, *Lamium maculatum*, with different numbers of visits by worker bumblebees (*Bombus pascuorum*), were analyzed by gas chromatography/mass spectrometry (GC/MS). Several odd-numbered alkenes, in addition to the plants' own cuticular lipids (mostly saturated alkanes), were identified. Pentacosenes (C25:1) were the clearest *B. pascuorum* markers. The quantity of pentacosenes in corolla washes increased positively and linearly with the number of visits that a flower had received. Furthermore, the amount of pentacosenes left on corollae did not change for two hours following the last bumblebee visit (Eltz 2006), thus suggesting that these bee lipids could serve as a long-term information store of bee visits.

In the present study, we further investigated this phenomenon by extending the time scale over which footprint retention is measured and by investigating the extent that retention is affected by environmental variables. We also conducted a field survey that tested the hypothesis that the amount of bumblebee footprint chemicals obtained from corollae of wild comfrey (*Symphytum officinale*) is indicative of the number of bumblebee visits. We show that the alkene amount on flower corollae can be used as a predictor of visit frequency, even in a natural, dynamic foraging environment.

Methods and Materials

All laboratory experiments were conducted in a climate chamber at the Department of Sensory Ecology at the University of Düsseldorf. *Bombus terrestris* colonies (Koppert Biological Systems) were maintained in a nest box (30×30×20 cm), which was connected to a feeding box (40×40×80 cm) via a Plexiglas tunnel (75 cm long). The colonies were fed with 20 ml sugar syrup (ApiInvert®) each day, provided in plastic syringes (5 ml). Pollen was supplied *ad libitum* directly into the nest box. The observation of bumblebee visits on flowers of wild comfrey (*S. officinale*) took place on the 29th July 2007 in pastures and meadows near Himmelgeist and Urdenbach, south of Düsseldorf.

Footprint Accumulation and Retention Under Different Ambient Temperatures Worker *B. terrestris* were allowed to forage on flowers of potted foxglove (*Digitalis grandiflora*; in 2007) or cowslip (*Primula veris;* in 2008) placed in the feeding box. Visits to individual flowers were recorded with the help of a computer and the software clbehave (Compulights 2005). To maintain high attractiveness of the flowers, small amounts of ApiInvert® were pipetted into the corollae at regular intervals.

The number of visits to individual flowers of *D. grandiflora* (2007) was manipulated and varied gradually between 31 and 51, whereas flowers of *P. veris* (2008) were allowed fixed numbers of 0, 20, or 40 visits. In both years, we tested how long the visited flowers retained deposited alkenes by taking corolla samples after 0, 6, and 24 h following the last bumblebee visit. The experiment was replicated under two different ambient temperatures (15 and 25°C) in both years, to test for effect of temperature on alkene retention. Individual flower corollae were removed from the receptacle with clean forceps, and anthers cut off at the base with scissors. Each corolla was extracted for 30 sec. in 500 µl *n*-hexane (p.a., Merck) containing 10 µg of 2-undecanone as an internal standard. The extracts were stored at 2°C until GC/MS analysis (see below). We also analyzed samples of bumblebee cuticular lipids. For this, we randomly sampled workers from the experimental colonies and cut off their tarsi at the proximal end of the femur. All six tarsi of an individual were combined and extracted in the same way as corollae.

We tested for effects of time since the last visit and ambient temperature on the amount of bumblebee-derived nonacosenes (C29:1) in corolla washes. For *D. grandiflora* (2007), in which the flowers had received varying numbers of visits, we performed an analysis of covariance (ANCOVA) in SPSS 15. Time (0, 6, and 24 h) and temperature (15 and 25°C) were specified as factors, and the number of visits received per flower (31–51) was the covariate. For *P. veris* (2008), we used an analysis of variance (ANOVA) and tested for effects of the factors, time (0, 6, and 24 h), temperature (15 and 25°C) and number of visits (0, 20, and 40), on the amount of nonacosenes.

Retention of Synthetic (Z)-9-Tricosene on Flowers Tricosenes are among the most dominant components in the cuticular lipids of bumblebees (e.g., Goulson et al. 2000). The (Z)-9-isomer has, among other hydrocarbons, been detected in footprint deposits of bumblebee workers (Schmitt et al. 1991; S. Witjes, unpublished data). Potted deadnettles were introduced into a climate chamber and habituated to an ambient temperature of 25°C. We applied 0.1 µl of (*Z*)-9-tricosene (Aldrich, Milwaukee, WI, USA), as a model compound, to unvisited flower corollae using a 5 µl Hamilton syringe. The syringe was connected to an assembly micrometer gauge (Holex, Munich, Germany) to facilitate adjustment of the exact volume. Corolla samples

either were taken immediately (0 h treatment), or after 24 or 48 h following application. Individual corollae were extracted for 30 sec. in 1.5 ml *n*-hexane. We performed ANOVA to test for effect of storage time on the amount of (*Z*)-9-tricosene in corolla extracts.

Footprint Accumulation on Flowers of Wild Comfrey Comfrey is a common perennial plant in pastures and meadows along the river Rhine, where it is frequently visited by local bumblebees for nectar and pollen. On the 29th July 2007, we recorded insect visits to flowers of 63 individual plants in 10-min.-intervals distributed evenly over the day from 800 to 1600 h (on average 5.5 intervals per plant, or 55 min of observation). Individual plants were chosen from a total of sixteen patches, and visit data were recorded synchronously by eight teams of two observers, each team switching back and forth regularly between patches and individual plants, so as to reduce the effects of time of day on counts per plant. Bumblebees were the only regular flower visitors (99% of visits), and the occasional visits by other insects (unidentified syrphid flies and solitary bees) were excluded from further analysis. We recorded the species of visiting bumblebee and calculated the average number of bumblebee visits received in 10-min observation intervals per flower and plant for each bumblebee species. At the end of the observation period, we randomly picked 5 flowers from each observed plant with clean forceps and extracted the corollae in 1 ml *n*-hexane (p.a., Merck) containing 10 µg of 2-undecanone as an internal standard for GC/MS analysis. We performed a linear regression analysis to test for an effect of the number of bumblebee visits on the amount of retained alkenes on flower corollae.

Chemical Analysis GC/MS analysis was performed with an HP 5890 II GC, equipped with a 30-m non-polar DB-5 column, connected to a HP 5972 mass selective detector, and an HP 7673 autoinjector (in splitless mode). The column oven was heated from 60 to 300°C at 3°C min^{-1}. Hydrocarbons were characterized by comparison of their mass spectra and retention times with that of authentic reference samples. For the purpose of the present study we did not differentiate between different alkene isomers, but pooled all alkene peaks of a given chain length.

Results

Footprint Accumulation and Retention under Different Ambient Temperatures Tarsal extracts of *B. terrestris* (*N*=10) contained *n*-alkanes and alkenes with chain length from 21 to 31, while corolla extracts of unvisited *D. grandiflora* (*N*=10) and *P. veris* (*N*=10) contained saturated alkanes, but no detectable amounts of unsaturated alkenes (Fig. 1). Nonacosenes (C29:1) were the most abundant class of alkenes on the tarsal cuticle of *B. terrestris* (Fig. 1) and, therefore, these chemicals were quantified as an indicator of bumblebee footprints. There were significant positive effects of the number of bumblebee visits on the amount of nonacosenes in corolla extracts of *D. grandiflora* in 2007 (ANCOVA: $N=77$; $df=1$; $F=9.664$; $P<0.05$) and of *P. veris* in 2008 (ANOVA: $N=180$; $df=2$; $F=69.018$; $P<0.001$) (Fig. 2). In neither case was there an effect of ambient temperature on the amount of nonacosenes in corolla extracts (*D. grandiflora*, $N=77$; $df=1$; $F<0.001$; N.S; *P. veris,* $N=180$; $df=1$; $F=1.074$; N.S) (Fig. 2). The amount of nonacosenes was not significantly affected by the time elapsed since the last visit to *P. veris* ($N=180$; $df=2$; $F=0.69$; N.S) (Fig. 2). In *D. grandiflora*, there was a marginal effect of time ($N=77$: $df=2$; $F=3.126$; $P=0.05$), with the amount of nonacosenes being, on average, reduced by 14% on corollae extracted 24 h, compared to 0 h, after the last visit.

Retention of Synthetic (Z)-9-Tricosene On Flowers No (*Z*)-9-tricosene was detected in extracts of unmanipulated control corollae of *L. maculatum* ($N=24$). In contrast, all treated corollae contained (*Z*)-9-tricosene (Fig. 3). There was no significant effect of the time since application on the amount of (*Z*)-9-tricosene on treated corollae (*ANOVA*: $N=57$; $df=2$; $F=0.358$; N.S.) (Fig. 3).

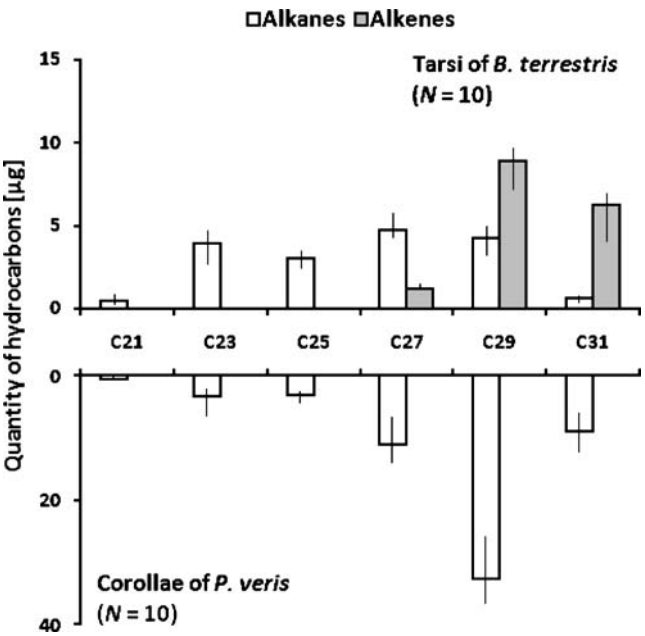

Fig. 1 Quantities of odd-numbered hydrocarbons in extracts of *Bombus terrestris* tarsi (µg/insect; $N=10$) and unvisited corollae (µg/corolla) of *Primula veris* ($N=10$), shown as medians with quartile ranges

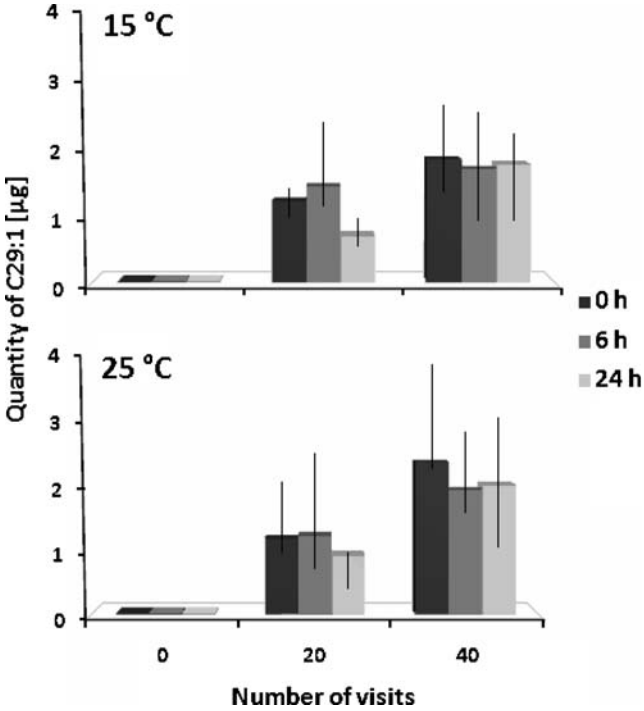

Fig. 2 Quantities (µg/corolla) of nonacosenes from *Primula veris* corollae, extracted 0, 6, or 24 h following the last bumblebee visit. The experiment was carried out at 15°C (*top*) or 25°C (*bottom*) ambient temperature. Corollae received 0, 20, or 40 visits by workers of *Bombus terrestris*. Median amounts with quartile ranges are shown

Footprint Accumulation on Flowers of Wild Comfrey The corollae of unvisited flowers of comfrey contained no detectable quantity of alkenes (S. Witjes, unpublished data). Workers of *B. pascuorum* were the most abundant visitors of comfrey at the time and contributed roughly 80% of all observed flower visits. The remaining 20% of visits were by *B. hortorum* (13 %), *B. terrestris* (4 %) and *B. pratorum* (3 %). The extracts of visited comfrey corollae contained alkenes of chain lengths from 21 to 31, corresponding well with that found in tarsal extracts of the visiting species of bumblebees (Goulson et al. 2000, Eltz 2006, S. Witjes, unpublished data). There was a significant positive relationship between the number of bumblebee visits observed per flower for a given plant during the observation intervals and the overall amount of alkenes on corollae of those plants (Fig. 4; Linear Regression: N=63; dF=62; F=32.83; P<0.001).

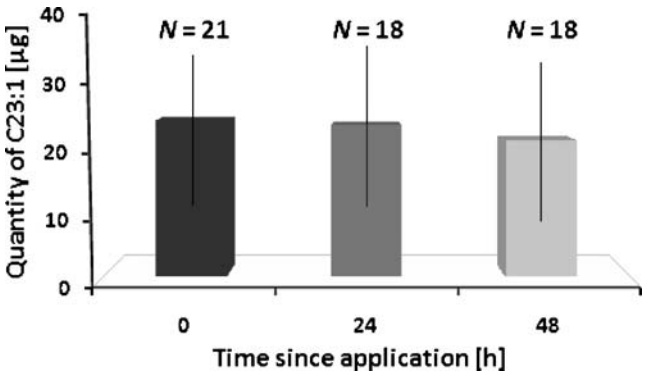

Fig. 3 Quantities (µg/corolla) of (*Z*)-9-tricosene from corollae of *Lamium maculatum* 0, 24, or 48 h after application. Data are shown as mean values with standard deviation

Discussion

This study provides further evidence that flowers retain a long-term chemical record of bumblebee visits. First, the amount of *B. terrestris*-derived nonacosenes washed from corollae was closely related to the number of bumblebee visits to the respective corollae in laboratory experiments. Second, the amount of marker alkenes remained unchanged over periods of 24 (footprint nonacosenes) to 48 (synthetic (*Z*)-9-tricosene) hours after the visits/manipulations, indicating that flower petals retain a quantitative record of bumblebee visits for a period similar to the lifetime of individual flowers of many temperate bee-pollinated plant species (Molisch 1929; Stead 1992). Third, the laboratory results were confirmed by our survey of wild comfrey plants, in which the amount of alkenes on flower corollae was closely related to the number of visits flowers of those plants received during the previous eight hours. Overall, our results indicate that alkene footprints on flower corollae can serve as an information source of bumblebee visits in natural populations of plants, especially since unsaturated alkenes seem to be absent or rare in epicuticular waxes of unvisited flowers (Griffiths et al. 1999, 2000; Goodwin et al. 2003; Eltz 2006). The alkene footprint, due to its cumulative nature, effectively integrates visitation dynamics over the entire exposure time of a flower, possibly providing a more accurate measure of bumblebee visits than an observational method, especially in studies with multiple replicates and limited observers.

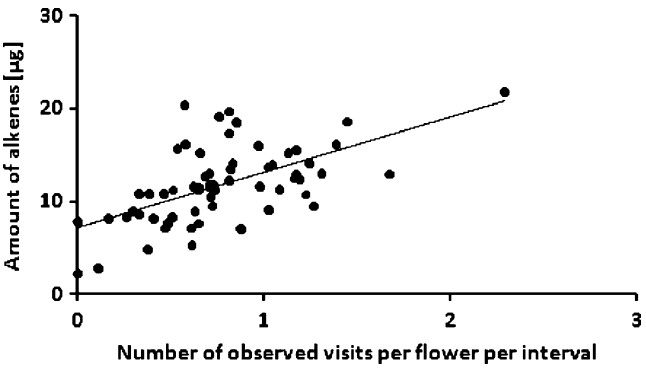

Fig. 4 Quantities of total alkenes on corollae of wild *Symphytum officinale* plants (N=63) in relation to the mean number of bumblebee visits the plants had received per flower in 10-minute observation-intervals during the day

Cuticular hydrocarbons are typically of low volatility, a point illustrated by a study that used combinations of synthetic alkanes (C24 to C31) to mark the elytra of milkweed beetles; these alkane profiles remained unchanged in quality and quantity over weeks despite exposure to direct sun and rain (Ginzel and Hanks 2002). The long-term retention of bee footprints on flowers may be promoted by the physicochemical characteristics of plant surfaces. Following deposition, bee hydrocarbons probably are integrated into the semi-liquid layer of plant cuticular waxes (Jetter et al. 2000; Eltz 2006), reducing their susceptibility to evaporation.

In agreement with Ginzel and Hanks (2002), bumblebee alkene retention was not influenced by changes in ambient temperature, at least over the temperatures (15 or 25°C) used in the experiments. The effects of more extreme temperature regimes or variation in exposure to direct sun were not investigated in detail in the present study. However, preliminary tests in an incubator oven suggest that evaporative losses of (*Z*)-9-tricosene droplets from filter paper are small even at 60°C (7.2 % over 24 h; S. Witjes, unpublished data). This suggests that variability of hydrocarbon retention should be low across a broad range of climatic conditions, thus allowing for comparisons among samples taken at different dates within the same general season/region.

It should be emphasized that the amount of hydrocarbons deposited on corollae of different plant species may vary substantially due to differences in flower morphology and in the way visitors contact corolla surfaces. Thus, each species of plant is likely to require calibration for determination of the number of visits. We currently are testing the applicability of footprint quantification as a tool to retrace the composition of the flower-visiting bumblebee community in wild populations of comfrey. Bumblebees show species-specific differences in hydrocarbon profiles (Goulson et al. 2000; Eltz 2006), and preliminary data indicate that those differences can be used to reconstruct the visiting bumblebee community (Witjes and Eltz, unpublished).

Quantification of hydrocarbon footprints on flowers may represent a cheap and reliable tool to quantify bumblebee visits in pollination studies, thus helping to reduce the problem of insufficient temporal and spatial replication in studies of pollinator decline.

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to produce latex, which serves as a physical barrier and a source of concentrated toxins effective against chewing herbivores (Dussourd and Eisner 1987; Zalucki and Malcolm 1999; Zalucki et al. 2001b; Agrawal 2005; Agrawal and Konno 2009). Latex accumulates under pressure in special cells (laticifers), which quickly release the sticky substance upon damage. By partially severing a leaf's petiole or cutting the midrib, the flow of latex to distal tissues in that leaf essentially can be stopped. This treatment substantially improved the performance of monarch caterpillars on four latex-rich milkweed species, but had relatively minimal effect on caterpillars feeding on four milkweed species that produce less latex (Zalucki and Malcolm 1999; Zalucki et al. 2001a, b). In addition, genetic families of *Asclepias syriaca* L. that exude more latex also have enhanced resistance to several insect species in feeding assays and in the field (Agrawal and Van Zandt 2003; Van Zandt and Agrawal 2004; Agrawal 2005).

All milkweed species also contain cardenolides, with concentrations varying over several orders of magnitude across species and among organs. Cardenolides inhibit Na^+/K^+-ATPases, which are important for maintenance of membrane potential in most animal cells. Cardenolides are toxic to a wide array of animals (Malcolm 1991). Differences in structure and polarity have been linked to differential absorption by animals, resulting in differential toxicity (Seiber et al. 1980; Malcolm 1991; Frick and Wink 1995). For example, the non-polar compound digitoxin is almost completely absorbed, irrespective of how it is administered to insects; conversely, ouabain, a highly polar cardenolide, is absorbed slowly from the gastrointestinal tract (Malcolm 1991). The Na^+/K^+ ATPase from specialist herbivores, such as the monarch butterfly (*Danaus plexippus*) has a slightly modified cardenolide binding site, thus reducing cardenolide toxicity (Holzinger and Wink 1996). Specialist herbivores of milkweed also may sequester cardenolides that are used for their own defense (Brower et al. 1972; Malcolm et al. 1989).

Other secondary metabolites that occur in milkweeds are cysteine proteases. Proteases, found in all living organisms, cleave proteins. Various types are found in the latex of plants that belong to a diversity of phylogenetic clades. For example, cysteine proteases are reported in latex of families such as Caricaceae, Moraceae, and Apocynaceae (Light et al. 1964; Sgarbieri et al. 1964; Arribére et al. 1998), and serine protease in Moraceae, Euphorbiaceae, Apocynaceae, Convolvulaceae (Arima et al. 2000; Tomar et al. 2008). Direct evidence for the involvement of cysteine proteases in plant resistance against herbivores came from experiments in which the toxicity of wild fig (*Ficus virgata*) against two generalist herbivores disappeared when E-64, a specific cysteine protease inhibitor, was painted on leaf surfaces (Konno et al. 2004). In previous work, we found that cysteine protease activity showed a 24-fold variation across the latex of 36 milkweed species (Agrawal et al. 2008).

It has been proposed that the carbon/nitrogen ratio (C/N ratio) of plant tissues is a significant regulator of susceptibility to leaf-eating insects (Behmer 2009). In particular, nitrogen may be the limiting factor for herbivores (Mattson 1980). Changes in C/N ratios in leaves might, therefore, be seen as an additional line of defense against folivorous insects.

The orchestration of multiple types of defenses in response to herbivore attack seems to be the key to 'plant immunity' (Howe and Jander 2008). Jasmonic acid plays a central role in coordinating the induction of the multivariate responses (Chini et al. 2007; Thines et al. 2007). Yet, we still know remarkably little about how different defensive traits are altered by herbivory, which traits are the most important for resistance, or how trait expression is coordinated. Closely related species that show divergent defensive strategies are especially interesting to compare because they can give us insight into how traits and their importance in resistance vary and potentially how they evolve.

In this study, we assessed variation of three plant defense related traits (latex, cardenolides, and proteases), one primary metabolism trait (C/N ratio), and one measure of resistance (larval growth of specialist monarch butterfly caterpillars) among three milkweeds (*Asclepias spp.*). We examined induction by monarch larvae or by treatment with jasmonic acid. Additionally, we sought to disentangle the impacts of two potentially potent resistance components, latex and cardenolides, on larval growth.

Methods and Materials

Plants and Growth Milkweeds of the genus *Asclepias* include about 130 species in North America, including Mesoamerica and the Caribbean, and six additional species endemic to South America (Woodson 1954; Fishbein et al. 2009). Within *Asclepias* is a monophyletic clade, series Incarnatae, with about 21 species (Fishbein et al. 2009) that we have been studying with regard to the evolution of latex and cardenolides (Rasmann et al. 2009). Preliminary experiments within this clade permitted us to choose three species (*A. angustifolia, A. fascicularis*, and *A. barjoniifolia*) that vary in their latex production, cardenolide production, and inducibility (Rasmann et al. 2009; Agrawal et al. 2009). *Asclepias angustifolia*, found mainly in Arizona (USA) and northern Mexico, and *A. fascicularis,* found throughout the west coast of the United States and Northern Mexico (Woodson 1954), exude relatively little latex and produce low levels of cardenolides. Latex production is induced in the latter following monarch herbivory. *A. barjoniifolia*, principally found on hot dry slopes in inter-Andean

valleys of Bolivia and Northern Argentina (David 2007), produces more latex and higher levels of cardenolides constitutively. Cardenolides (Rasmann et al. 2009), but not latex (S.C. Cook et al., unpublished) are induced by monarch herbivory.

For all experiments, seeds were germinated at room temperature after being stratified at 4°C on moist filter paper for 2 wk. Seedlings were randomized and grown in potting soil (10 cm diam pots) in a growth chamber (14 h daylight, 26°C day: 20°C night). Plants were fertilized (N:P:K 21:5:20, 150 ppm N [μg/g]) once, 1 week after planting.

We used larvae of the monarch butteriflies *Danaus plexippus plexippus*, a species that feeds almost exclusively on *Asclepias* spp. Monarchs are native to North and South America, and are split into a subspecies in South America, *D. plexippus erippus* (Ackery and Vane-Wright 1984). We assume that the three *Asclepias* spp. that we studied are experiencing herbivory from these two subspecies of monarchs regularly.

Induction Experiments After 4 wk of growth, plants from the 3 species were divided equally into 3 groups: 1) controls, 2) plants induced by addition of one first instar monarch caterpillar, and 3) plants sprayed with roughly 1.5 ml of 0.5 mM jasmonic acid (N=16 plants per treatment, per species). After 4 d, all larvae used for induction were removed from the treated plants, and half of the plants (N=8 plants per treatment, and per species) were sampled to determine latex exudation and chemical composition (see below). The remaining plants were left untouched for 5 d before inoculating each with one first instar monarch caterpillar. This set of larvae was weighed (fresh weight) after 5 d of feeding to obtain a measure of larval growth rate.

Latex Exudation We measured the amount of latex exuded onto a 1 cm diam pre-weighted filter paper, after cutting the first 5 mm off the tip of the youngest fully expanded and intact leaf. Latex stopped flowing after ≈10 s, and all latex was absorbed on the filter paper. This disc was placed in a pre-weighed microcentrifuge tube and weighed to estimate wet latex exudation per plant. Our method is a reproducible assay for determining latex exudation.

Cysteine Protease Activity To measure protease activity, we prepared a 0.6% latex solution by removing 1–3 leaves at the petiole of each plant to collect 1.5 μl of latex, which was mixed with 250 μl of 50 mM sodium phosphate buffer (pH 7). We employed a modification of the spectrophotometric protocol developed by Konno et al. (2004). Briefly, a reaction buffer with casein (Sigma, St. Louis, MO, USA) as the substrate was incubated for 30 min and stopped with trichloroacetic acid. After precipitating the undigested casein, absorbance of the supernatant at 280 nm was recorded.

HPLC Analysis of Cardenolides After collecting latex, we harvested all aboveground plant tissue and immediately froze it at −80°C. Collected plant material was oven-dried at 50°C for 3 d and ground. Fifty milligrams of ground material, spiked with 20 μg of digitoxin (Sigma) as internal standard, was extracted with 1.9 ml of 95% ethanol in a sonicating water bath at 55°C for 20 min. Ethanol was evaporated, and the residue was dissolved in 0.5 ml methanol. Samples were analyzed by HPLC using a Gemini C18 reversed phase column (3 μm, 150×4.6 mm, Phenomenex, Torrance, CA, USA) and an Agilent 1,100 series instrument with diode array detection. The 15 ul injection was eluted at a constant flow of 0.7 ml/min with a gradient of acetronile and 0.25% phosphoric acid in water as follows: 0–5 min 20% acetonitrile; 20 min 70% acetonitrile; 20–25 min 70% acetonitrile; 30 min 95% acetonitrile; 30–35 min 95 % acetonitrile. Peaks were detected by a diode array detector at 218 nm, and absorbance spectra were recorded from 200 to 400 nm. Peaks showing a characteristic symmetrical absorption band with a maximum between 217 and 222 nm were recorded as cardenolides (Malcolm and Zalucki 1996). Concentrations were calculated and standardized by using a standard curve that related peak areas to known digitoxin concentrations.

Above-ground tissue C/N was assessed with ≈3 mg of dried and powdered material at the Cornell University Stable Isotope Laboratory.

All statistical analyses were conducted by using JMP (Version 7. SAS Institute Inc., Cary, NC, 2007). First, we performed an overall MANOVA on the four response variables together (latex, cardenolides, C/N ratio, and proteases). Then, we performed a two-way ANOVA on each of the responses to assess effects of species, treatment, and their interaction.

Artificial Diet Experiment To test specifically for the impacts of latex and cardenolides, we focused on one species (*A. angustifolia*). This species was chosen because it exudes comparatively small amounts of latex and has low levels of cardenolides. The species is relatively poorly induced by herbivory (Rasmann et al. 2009). We investigated the role of latex by comparing monarch larval weight gain on 5–wk-old plants grown as described above (treatment *Intact*) to weight gain of larvae fed on cut leaves (treatment *Cut*) (i.e., no latex exudation). All cut leaves were placed on moist filter paper in 10 cm diam plastic boxes, with the petiole in moistened foam to reduce desiccation. Moreover, we tested the effect of two cardenolides (digitoxin and ouabain) on larval growth by placing one first instar monarch larva on a cut leaf that was painted with either 0.5% dry weight (DW) digitoxin (Sigma) in a methanolic solution (treatment *Digitoxin*), or 0.5% DW ouabain (Sigma) in a methanolic solution (treatment *Ouabain*), or pure

methanol (treatment *Methanol*). All five treatments were randomized on a laboratory bench. Larvae were allowed to feed for 2 d before recording their mass. To assess the amount of leaf tissue eaten during the trial, we scanned the leaves and quantified the area of tissue consumed using ImageJ 1.41 software (http://rsbweb.nih.gov/ij/). Area was plotted against a known leaf mass per area regression curve (r^2=0.969, leaf mass=0.027* leaf area—0.01, P<0.001) to obtain the fresh leaf mass eaten by the insect. The effect of different treatments was assessed with one-way ANOVA. We assessed differences within species and treatments using Student's *t post-hoc* tests.

Results

Induction Experiment Overall, we found strong variability across the four plant traits (latex, cardenolides, C/N ratio, and proteases) measured across three species, and three treatments (Fig. 1, Table 1, MANOVA, species: Wilks' λ=, $F_{16,263.37}$=31.618, P<0.001; treatment: Wilks' λ=, $F_{8,172}$= 3.979, P<0. 001, interaction: Wilks' λ=, $F_{32,318.75}$=1.877, P<0.001). *Asclepias angustifolia* was the best host plant for monarch larvae, supporting an average growth rate (over 3 treatments) that was five-fold higher than the average rate on *A. barjoniifolia*, and three-fold higher than the average rate on *A. fascicularis*. Of the three species, only *A. fascicularis* showed increased resistance after induction by monarchs or jasmonic acid, and larvae on induced plants grew 68 and 74% less, respectively, compared to larvae on controls. In contrast, on *A. angustifolia* we found induced susceptibility; larvae grown on monarch-treated plants were 61% heavier compared to control plants. This effect was not mirrored in the jasmonic acid treatment, which had no effect (Fig. 1, Table 1). *Asclepias barjoniifolia* was uniformly of poor quality, and larval growth rate was not affected by either type of induction.

Latex exudation largely mirrored the effects of treatments and species on larval mass. *Asclepias barjoniifolia* and *A. fascicularis* produced the same amount of latex, while *A. angustifolia* produced four-fold less. *Asclepias barjoniifolia* increased latex production 32% following the jasmonic acid treatment, but production diminished more than 90% after monarch herbivory. Interestingly, after monarch damage, *A. barjoniifolia* latex production was essentially the same as *A. angustifolia*. Induction of latex also was found for *A. fascicularis,* with plants exuding 30% more following jasmonic acid or monarch induction.

Cardenolides were two and five times more abundant in *A. fascicularis* and *A. barjoniifolia,* respectively, than in *A. angustifolia.* A significant (30%) increase in cardenolide prosecution was induced in *A. barjoniifolia* by either induction treatment Overall the level of cardenolides was correlated with the number of individual cardenolide peaks found in our HPLC analysis (Pearson correlation: N=9, r^2=0.997, P<0.001). We found more peaks in *A. barjoniifolia* (8-10) than in *A. angustifolia* (4-6) or *A. fascicularis* (4-6) (Fig. 2). More cardenolide peaks were recorded after jasmonic acid or monarch treatments in all species. Across species, cardenolide peaks eluted between 15 and 20 minutes. Only *A. barjoniifolia* produced more polar cardenolides that eluted before 15 minutes (Fig. 2).

The C/N ratio was different across species, with *A. barjoniifolia* and *A. fascicularis* having a 22 and 14% higher ratio, respectively, than *A. angustifolia* (Fig. 1). Interestingly, jasmonic acid treatments decreased the ratio by 11%; but overall, monarch treatment increased it by 8%, resulting in a significant difference between these two treatments. Monarch treatment increased the ratio in *A. barjoniifolia* by 27%.

Proteases showed a significant species effect with *A. fascicularis* having almost twice the level found in *A. angustifolia,* and *A. barjoniifolia* having intermediate levels. (Fig. 1, Table 1). Jasmonic acid or monarch treatments generally decreased protease activity, with a dramatic 80% decrease in either jasmonic acid- or monarch-induced *A. barjoniifolia* compared to controls.

Artificial Diet Experiment Larval weight gain after two days of feeding was differentially affected by treatments ($F_{4,75}$= 4.68; P =0.002, Fig. 3a). Larvae were 1.7 times heavier on cut leaves, compared to those feeding on intact plants, indicating a role for latex in resistance to monarchs. However, larvae feeding on cut leaves painted with digitoxin performed as poorly as those feeding on intact plants. There was no effect of ouabain or the methanol control when painted on leaves, compared to control cut leaves.

The weight gain per amount of leaf mass eaten (food conversion efficiency) on digitoxin and on ouabain painted leaves was 38 and 36% lower than on control cut leaves, respectively ($F_{3,40}$=2.857, P=0.049, Fig. 3b). In other words, although conversion efficiency was decreased substantially for monarchs that consumed ouabain, they apparently were able to compensate for this reduction by consuming 53 % more tissue than larvae feeding on digitoxin.

Discussion

Variability of Responses Within plant taxa and across our three treatments, there was substantial variation in the constitutive and induced levels of several putative defense traits. Latex and cardenolides levels were species specific, and induction by monarchs and jasmonic acid had overlapping and distinct effects. Latex and cardenolides showed some correlation with larval performance. For instance, the

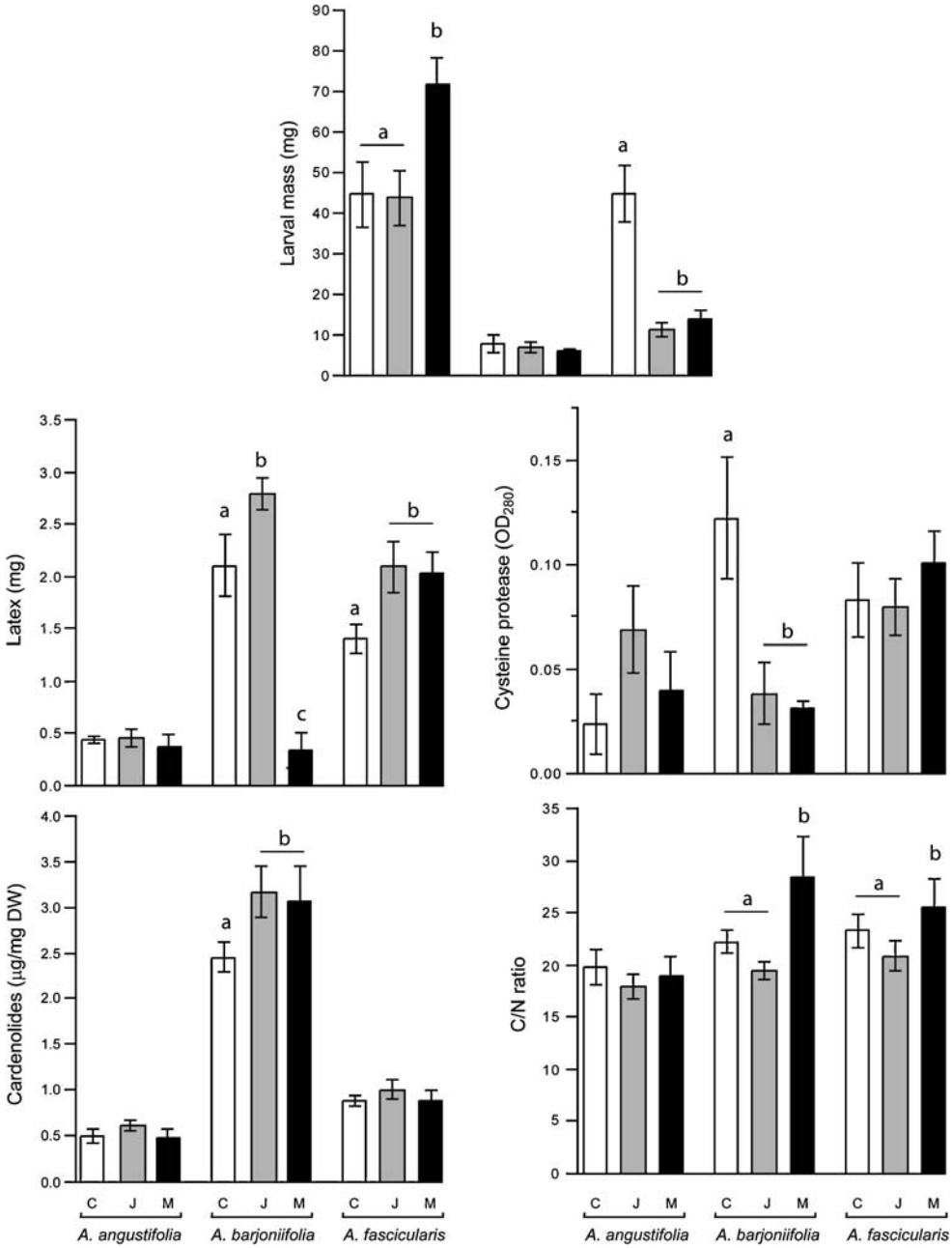

Fig. 1 Means ± SE of larval mass, latex, cardenolides, C/N ratio and proteases, in *Asclepias angustifolia*, *A. barjoniifolia*, and *A. fascicularis* and across the control (C), jasmonic acid (J), and monarch (M) treatments. Different letters above bars indicate significant differences among treatments within plant species ($P<0.05$)

low latex and low cardenolide species *A. angustifolia* supported more larval weight gain compared to high latex (*A. barjoniifolia* and *A. fascicularis*) or high cardenolide (*A. barjoniifolia*) species. The induction of latex but not cardenolides in *A. fascicularis* was accompanied by a decrease in larval weight gain (Fig. 1). In *A. barjoniifolia*, monarch induction did not alter larval growth because although cardenolides were higher after induction, latex production was diminished.

A high carbon to nitrogen ratio (C/N) could be caused by low levels of nitrogen or high levels of carbon-based compounds in the leaves, and in general indicates poor leaf quality. If a leaf is of a poor nutritive quality, it is predicted to be less defended than a high quality leaf (Coley 1983). In our case, we found species differences in the constitutive C/N ratios, but these were not mirrored by cardenolide levels. These differences in C/N may be more influenced by abiotic factors that shape plant ecophysiology (Agrawal et al. 2009) than by a response to herbivory. Matsuki and Koike (2006) showed that across seven species of deciduous broad-leaved trees that differed in their leaf life span, C/N correlated negatively with total N and herbivore survival, revealing that older leaves with low nitrogen content were less suitable for caterpillar growth. Following monarch feeding on *A. barjoniifolia* and *A. fascicularis*, C/N increased; this could be a result of metabolite reallocation and changes in

Table 1 Overall univariate anovas testing the effects of species (*asclepias angustifolia, a. fascicularis,* and *a. barjoniifolia*), treatments (control, jasmonic acid, and monarch), and their interaction for each of the tested traits sampled in the induction experiment

Source	Effect	DF	*F* ratio	*P*
Larval mass	Species	2,44	55.860	**<0.0001**
	Treatment	2,44	4.503	**0.017**
	SxT	4,44	7.748	**<0.0001**
Latex	Species	2,58	59.533	**<0.0001**
	Treatment	2,58	16.082	**<0.0001**
	SxT	4,58	13.845	**<0.0001**
Cardenolides	Species	2,53	145.161	**<0.0001**
	Treatment	2,53	3.760	**0.03**
	SxT	4,53	1.780	0.147
No. cardenolide peaks	Species	2,53	179.678	**<0.0001**
	Treatment	2,53	5.705	**0.006**
	SxT	4,53	2.076	0.097
C/N	Species	2,52	6.533	**0.003**
	Treatment	2,52	5.028	**0.011**
	SxT	4,52	0.969	0.432
Proteases	Species	2,53	3.884	**0.027**
	Treatment	2,53	0.682	0.510
	SxT	4,53	3.810	**0.009**

Bold indicates significant values ($P<0.05$).

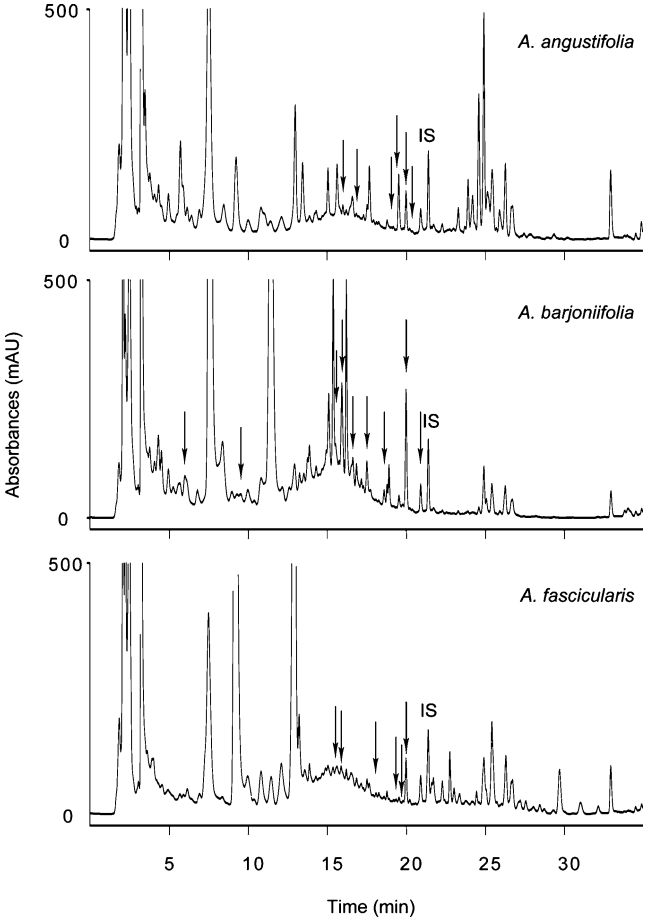

Fig. 2 Sample HPLC chromatograms of peak absorbance at 218 nm of leaf extracts for the three species of *Asclepias* studied. Scale is truncated at 500 mAU for better visualization of cardenolide peaks. Arrows mark all cardenolides found in undamaged plants. IS marks the internal standard (digitoxin)

photosynthesis. Consistent with the current findings, Agrawal and Fishbein (2006) showed that across 24 species of *Asclepias,* C/N correlated with leaf toughness, but not with qualitative defenses such as latex, cardenolides, or trichomes.

Cysteine proteases in latex drastically reduce herbivore growth (Konno et al. 2004). Across 36 species of *Asclepias,* Agrawal et al. (2008) showed that cysteine proteases in latex correlated positively with the amount of latex produced by the plant, but traded off with cardenolide concentration in latex. Here, the species with the highest leaf cardenolide level, *A. barjoniifolia*, also had the highest protease activity. We speculate that the consistently poor quality of *A. barjoniifolia,* irrespective of induced plant responses, was due to the high cardenolide, high latex, and high protease content of the species.

In the swamp milkweed *A. incarnata,* there was no influence of reduced latex flow on growth rates or survival of first-instar monarchs (Zalucki and Malcolm 1999). This is both a low latex and low cardenolide plant species, with smooth, soft, lanceolate leaves. The lack of a response to reduced latex flow in a low-latex and low-cardenolide milkweed lends credence to hypothesis that both latex and cardenolides are integrated defenses against early-stage monarch larvae. Similarly, Zalucki et al. (2001b) found that monarch larvae on nine *Asclepias* species generally grew faster and survived better on leaves when latex flow was reduced by partial severance of the leaf petiole. The outcome depended on milkweed species, and was related to the amount of latex and cardenolides produced, as well as to other plant characters, such as leaf hairs and microclimate. They concluded that several other parameters of plant quality/defense must play an important role in larval performance. We agree with this conclusion based on the three *Asclepias* species studied here.

Specificity of Induction Jasmonic acid now is widely accepted to be a key intracellular signal in mediating responses to insect attack (de Bruxelles and Roberts 2001), eliciting both direct and indirect defenses resulting in lower herbivore performance (Thaler et al. 2002). Here, we showed that exogenous application of jasmonic acid resulted in species- and trait-specific responses, which were often different from the responses plants produced after caterpillar feeding (Fig. 1). For example, latex induction

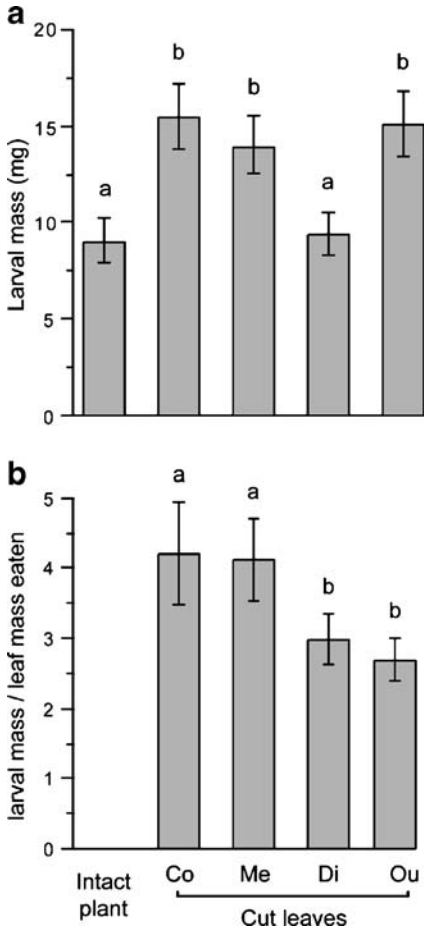

Fig. 3 **a**) Mean ± SE larval mass after 2 days feeding on *Asclepias angustifolia* intact plants, cut leaves (Co), cut leaves painted with methanol (Me), cut leaves painted with digitoxin (Di), or on cut leaves painted with ouabain (Ou). **b**) Mean ± SE conversion efficiency for each treatment other than the intact plant. Different letters above bars indicate significant differences among treatments ($P<0.05$)

was the same after jasmonic acid or monarch herbivory in *A. fascicularis*, but not for *A. barjoniifolia*.

One might think that the "sabotaging" behavior of monarch caterpillars, which notch veins and cut small moats through the leaves that reduce latex exudation at the feeding sites (Dussourd and Denno 1991; Zalucki and Brower 1992), was the cause of the reduced latex flow in *A. barjoniifolia.* However, in this species, latex was collected from healthy leaves, often opposite to the damaged one. Additionally, reduced latex production has been noted in other experiments with *A. barjoniifolia* when latex was collected several days after monarch feeding had ceased (S.C. Cook et al., unpublished data). Thus, the decrease in latex production in *A. barjoniifolia* following herbivory suggests that there may be other factors than mechanical sabotage that are involved in reducing latex production. Jasmonic acid, on the other hand, increased latex in the same species. This leads us to speculate that monarch larvae can impede or even disrupt some aspects of jasmonic acid induction in *A. barjoniifolia*.

Separating the Effects of Different Defense Components

Latex and cardenolides have detrimental effects on the growth rate of first-instar monarch larvae (Zalucki et al. 2001a, b; Agrawal 2005). Our results confirm this observation as we found that larvae fed on cut leaves perform better than larvae fed on cut leaves painted with digitoxin, or on intact plants. Based on field observations of naturally laid eggs, Zalucki et al. (1990) showed that survival in the first instars was weakly negatively correlated with plant cardenolide concentration in *A. humistrata*. Zalucki and Brower (1992) subsequently confirmed this observation experimentally and suggested that some of the high mortality in instars might be related to the cardenolide concentration in latex, or to the latex itself. This was confirmed by manipulative experiments in the field. By notching the basal side of the mid-vein of a leaf, Zalucki et al. (2001a) reduced latex flow and increased monarch larval growth rate. In our experiment, by removing the leaf from the plant, we similarly reduced latex exudation, thus also favoring larval growth. Here, however, we cannot completely exclude alterative explanations for the reduced resistance, such as the lack of responses that involves changes in systemic resource allocation.

Various levels of several cardenolides are found in latex and all other parts of the plant (Malcolm 1991), Fig. 2). Malcolm (1991) suggested that different types of cardenolides, having different polarities, are absorbed at different rates from the insect gut (Frick and Wink 1995). We compared the impacts of two cardenolides with different polarities. Larvae feeding on cut leaves painted with digitoxin (less polar) grew more poorly then larvae growing on leaves painted with ouabain (more polar). Although our conclusions regarding cardenolide toxicity to monarchs based on digitoxin and ouabain are tentative (digitoxin is only found in *Digitalis* sp.), our results are consistent with the prediction that non-polar compounds are more readily absorbed than polar ones (Wright 1960; Duffey and Scudder 1974; Malcolm 1991). The species studied here have cardenolides that may have polarities similar to digitoxin, based on elution time on reversed phase HPLC. *Asclepias barjoniifolia* also had some cardenolides that may have been more polar (Fig. 2).

In summary, although it is convenient to consider plant defense as a single trait, plants typically utilize a broad arsenal of defensive traits against herbivores (Duffey and Stout 1996; Romeo et al. 1996). Even when a plant species apparently is defended by a single type or class of defense chemical (like latex or cardenolides in our case), typically there are many specific forms of those compounds (Berenbaum et al. 1986; Malcolm 1991; Bennett and

Wallsgrove 1994). Thus, it is more useful to think about plant defense as a suite of traits, which might include aspects of a plant's nutritional quality, physical characteristics, toxicity, phenology, regrowth capacity, and indirect defense (Agrawal and Fishbein 2006). Synergistic interactions between multiple traits are particularly important in providing a greater level of defense than would be possible if the traits were present independently (Broadway et al. 1986; Berenbaum et al. 1991; Rasmann and Agrawal 2009).

By studying closely related species, we have taken account of similarities due to shared ancestry, and have attempted to identify important differences in defensive features (Agrawal and Fishbein 2006). Interestingly, our closely related species were substantially different in defensive traits, suggesting that they may have evolved under dramatically different habitats and herbivory regimes. An additional dimension to the plant defense syndrome hypothesis would be to incorporate induction and repression of defensive traits following herbivore attack. We found that *A. angustifolia* is nearly defenseless, so we suggest that for this species induction would not be helpful because herbivores generally have high performance on this species. On the contrary, *A. barjoniifolia* is extremely toxic and induced responses seem to be redundant, not providing additional protection. Finally, *A. fascicularis* had intermediate traits, with inducible latex, which in coordination with the other components of the defensive system, promoted reduced larval growth.

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pollinators and seed dispersers (Dudareva et al. 2004). Herbivore-induced plant volatiles (HIPVs) currently are the most intensively studied VOCs in the field of chemical ecology. A wide range of plant species has been shown to emit new VOCs or increased amounts of constitutive VOCs following herbivore damage (Dicke et al. 2003; Unsicker et al. 2009). Nevertheless, whether or not these volatiles are a result of co-evolutionary processes among plants, herbivores, and their natural enemies remains unclear (Holopainen 2004). This is because most studies have been performed on agriculturally important plant species under greenhouse or laboratory conditions, thus making it difficult to derive direct conclusions on the evolutionary role of HIPVs (Arimura et al. 2005). Although greenhouse and laboratory studies provide useful information on individual interactions among plants, herbivores, and natural enemies, they often fail to include various biotic and abiotic stresses that influence volatile production under natural conditions. Plants in the field often are attacked by multiple insect herbivores and diseases at the same time, in contrast to the situation in the laboratory where plant damage is carefully controlled and usually restricted to one attacking species. Abiotic stresses, such as high light and drought also have been shown to influence plant VOC production although these are rarely studied in the laboratory (Gouinguene and Turlings 2002; Blanch et al. 2007). There are only a few studies that report the volatile bouquets for the vegetative parts of naturally growing plants, for instance those on *Nicotiana attenuata* (Kessler and Baldwin 2001), wild lima bean (Heil 2004), or *Solidago altissima* (Tooker et al. 2008).

In the present study, we investigated HIPV emission from the legume *Trifolium pratense* L. (red clover), under both laboratory and field conditions. Red clover is a common forb in many Central European and North American grassland ecosystems. Although it is cultivated as a forage crop and used to increase soil nitrogen in many parts of the world, it remains a common species of natural meadow plant communities (Hoghjensen and Schjoerring 1994; Andrews et al. 2007). The nitrogen-fixing function of nodules found on red clover plays a key role in natural grassland ecosystems where other plant species growing near such legumes benefit from the nitrogen released from root exudates or upon plant death (Spehn et al. 2002; Temperton et al. 2007). Considered one of the key species in grassland ecosystems, red clover provides a useful basis for looking at multiple ecological roles of HIPVs since it is attacked by various herbivores. In cultivated stands, the root borer *Hylastinus obscurus* (Marsham) and the clover root weevil (*Sitona lepidus* Gyllenhal) both have been reported to cause extensive damage in pastures (Tapia et al. 2005; Murray et al. 2007). In natural grasslands, the level of damage on *T. pratense* was reported to be affected by multiple factors including plant community biomass, plant species richness, and plant species composition (Scherber et al. 2006; Unsicker et al. 2006).

Early studies on the induced defenses of *T. pratense* focused on defense against pathogens. Several authors showed that *T. pratense* produces various secondary metabolites such as pterocarpanoid phytoalexins and amides in response to fungi (Macfoy and Smith 1979; Mcmurchy and Higgins 1984). Jasmonic acid (JA) was shown to induce the production of caffeoyl DOPA (clovamide) in the roots but not in the shoots of *T. pratense* (Tebayashi et al. 2000). Later studies focused on the attraction of root herbivores to root volatiles in *T. pratense* from root extracts (Tapia et al. 2007). Other research on the volatile organic compounds of *T. pratense* has used dried or fresh leaf extracts to determine the quality of the hay produced (Figueiredo et al. 2007).

In the present study, we investigated: 1) the VOCs emitted by *T. pratense* under laboratory conditions after caterpillar herbivory and mechanical wounding; 2) the effects of temperature and sampling duration on VOC composition in the laboratory; and 3) the emission of herbivore-induced volatiles under field conditions.

Methods and Materials

VOC Production of *T. pratense* Under Laboratory Conditions

Plants and Insects Ramets of *T. pratense* were transplanted from the field in April 2007 when plants were still small (<10 cm in height). Plants were trimmed back to 3 cm, potted in 16 cm pots with a mixture of compost soil and sand (2:1), and allowed to grow under greenhouse conditions (day:night temperatures of 22–25°C: 18–20°C, 16/8 h L/D cycle, photosynthetically active radiation ca. 180 µmol m^{-2} s^{-1}, and 30–55% relative humidity). Measurements of volatiles were performed as soon as plants reached an average height of 15 cm (14 days after they were trimmed).

Caterpillars of Spodoptera littoralis Boisd (Lepidoptera: Noctuidae), a generalist leaf-chewing herbivore, were hatched from eggs obtained from Syngenta (Basel, Switzerland) and reared on agar-based artificial media (Fontana et al. 2009) at 23–25°C with a 16/8 h L/D cycle until they reached the 3rd instar.

Herbivory and Temperature Manipulations To determine the effect of herbivory on *T. pratense,* we compared volatile emissions of plants damaged by *S. littoralis* to emissions of control plants and plants subjected to mechanical damage. In total, there were 6 treatments: mechanical wounding, herbivore feeding (4 treatments) and a control. Control

plants were undamaged and not exposed to leaf herbivores prior to the experiment. Mechanical wounding was induced 15 min before the start of volatile collection using a commercially available fabric pattern wheel (McCloud and Baldwin 1997). Four tracks were made on the leaf lamina parallel to the mid-rib on 4–6 leaves of the plant depending on its size. For the feeding treatments, plants were exposed to three *S. littoralis* larvae previously starved for 8 h for a period of: a) 30 min; b) 2 h; c) 8 h; and d) 24 h before the start of volatile collection. The larvae were removed from the plants at the end of each feeding treatment immediately before volatiles were collected.

The effects of temperature on VOC emission were tested by performing experiments at both 20°C and at 30°C. We used three replicates for each temperature-herbivory treatment combinations so that 36 plants were measured in total (6 treatments x 2 temperatures x 3 replicates)

We also were interested in determining the effect of sampling time on volatile detection. Consequently, for each temperature-herbivory treatment combination, three measurements were performed: 15 min, 30 min, and 4 h. These sampling time tests were carried out consecutively; entrainment vials were opened at each time point to change the traps. This gave a total of 3 pseudo-replicates per temperature-herbivory combination.

Volatile Collection in the Laboratory VOCs were collected using a dynamic headspace sampling system that was installed in a climate chamber under controlled conditions (70% relative humidity and 150 μmol m^{-2} s^{-1} PAR) as described previously (Chen et al. 2003). The plants were placed inside 3 l glass desiccators (Schott, Jena, Germany). Each desiccator was closed tightly with a glass lid and equipped with a valve through which charcoal-purified air entered the system at 4 l min^{-1}. Volatiles were collected from the plants by passing the outlet air through a super-Q filter (containing 50 mg Super-Q absorbent) (Analytical Research Systems, Gainesville, FL, USA) at a rate of 2 l min^{-1}; the excess was released through vents at the top of the glass jars. VOCs from 6 *T. pratense* plants were collected simultaneously in the volatile collection chamber. Replicates were made on different days but at the same time of day, from 0900 to 1500 h. After each collection, the volatiles were eluted from the traps with 150 µl hexane and 10 µl of an internal standard (n-nonylacetate; Sigma-Aldrich, Seelze, Germany) at a concentration of 4 ng μl^{-1}. At the end of each VOC collection, the leaves per plant were counted and percent herbivory was estimated.

Volatile Analysis Sample analysis was conducted using an Agilent 6890 Series gas chromatograph, with helium as the carrier gas; the outlet of the column (DB-5MS 30 m× 0.25 mm×0.25 µm film) was coupled to a mass spectrometer (Agilent 5973 N quadrupole detector). Parameters for electron impact sample ionization were as follows: interface temperature, 280°C; repeller, 30 V; emission, 34.6µA; electron energy, 70 eV; source temperature, 230°C. The gas chromatographic conditions were as follows: splitless injection at 220°C, initial oven temperature, 40°C for 3 min, increased at 5°C min^{-1} to 210°C followed by an increase of 60°C min^{-1} to 300°C and held for 2 min. To identify compounds, the mass spectra were compared with those in the commercially available mass spectra libraries NIST and Wiley. Individual compounds were quantified by calculating the peak area relative to the peak area for the internal standard. Reference factors were determined using a mixture of pure standards (where available, see Tables 1, 2 and 3). For statistical analysis, all volatile emissions were standardized to the amount of compounds released per leaf (ng $leaf^{-1}$ h^{-1}). Dry weight (DW) of leaves of *T. pratense* was in later experiments found to range between 0.01–0.04 g $leaf^{-1}$.

VOC Production of *T. pratense* Under Field Conditions

Design of Field Sampling Field measurements were carried out in plots of "The Jena Experiment" (a long term grassland biodiversity experiment established in 2002 in Jena, Germany; for details, see Roscher et al. 2004). The plant communities were established from seeds in 2002 from a pool of nine plant species (the so-called dominance experiment, Roscher et al. 2004): *Trifolium pratense*, *Trifolium repens*, *Anthriscus sylvestris*, *Geranium pratense*, *Alopecurus pratensis*, *Arrhenatherum elatius*, *Dactylis glomerata*, *Phleum pratense*, and *Poa trivialis*. Plant species composition has been maintained by weeding since the establishment of the plots in 2002. We randomly selected seven 3.5×3.5 m plots, all with three-species mixtures, in which to perform plant volatile measurements on the target species, *T. pratense*. Two *T. pratense* plants were selected from each plot, one with high levels of leaf herbivory (based on a visual estimate of leaf area loss due to chewing insects) and one with low levels of herbivory. VOCs from both plants were collected simultaneously, after which the plants were harvested and the leaf area consumed by herbivores was immediately assessed visually on five randomly selected leaves per plant to the nearest 5% for each leaflet. The mean percent damage value of the 5 leaves was used as the percent damage of the plant for further analysis. Finally, leaf dry weight was determined after plants were dried for 48 h at 70°C.

Volatile Collection and Analysis The volatile collection consisted of a push-pull system (Tholl et al. 2006). Plants in the field were enclosed in a 30×50 cm polyethylene terephthalate (PET) cooking bag (Toppits® Bratschlauch,

Table 1 Volatile emission from *Trifolium pratense* in the laboratory experiment (mean ± SE amount, ng leaf^{-1} h^{-1}) emitted after 4 hours of sampling at *20°C*

Compounds	Control	Mechanical Wounding	**Herbivory treatments**			
			30min	2h	8h	24h
α-Pinene[d]	0.11±0.11	0.07±0.07	0.11±0.11	0.16±0.16	0.08±0.08	–
Sabinene[d]	0.04±0.04	–	0.1±0.1	–	–	–
β-Pinene[d]	0.09±0.09	0.1±0.1	–	0.27±0.27	–	–
Limonene*[d]	0.11±0.11	0.11±0.11	0.13±0.09	0.18±0.18	0.14±0.07	0.07±0.07
1,8-Cineole[d]	–	–	0.39±0.39	–	–	–
(*Z*)-β-Ocimene[d]	**–[a]**	**0.38±0.21[a]**	**0.55±0.27[ab]**	**1.6±0.34[b]**	**4.11±2.39[b]**	**5.71±3.13[b]**
(*E*)-β-Ocimene[d]	**0.06±0.06[a]**	**1.66±0.84[a]**	**2.75±1.19[a]**	**6.93±1.42[b]**	**19.68±11.19[bc]**	**26.21±12.55[c]**
Linalool[d]	**–[a]**	**–[a]**	**–[a]**	**0.36±0.19[b]**	**0.26±0.26[b]**	**1.82±1.58[c]**
Indole	0.13±0.13	0.29±0.29	0.6±0.36	0.15±0.15	0.15±0.07	2.74±2.67
(*Z*)-Jasmone[d]	0.06±0.06	–	–	0±0	0.14±0.14	1.08±1.08
(*E*)-β-Caryophyllene[d]	**–[a]**	**–[a]**	**0.01±0.01[b]**	**0.51±0.17[b]**	**0.38±0.19[b]**	**3.17±2.49[b]**
(*E*)-β-Farnesene[d]	0.05±0.05	–	–	0.1±0.1	0.31±0.31	–
Germacrene D	0.27±0.27	–	–	–	–	–
(*E,E*)-α-Farnesene*[d]	**–[a]**	**0.15±0.15[a]**	**–[a]**	**–[a]**	**0.12±0.06[b]**	**0.48±0.28[b]**
DMNT	**–[a]**	**–[ab]**	**0.51±0.43[ab]**	**0.47±0.28[ab]**	**2.88±2.5b[c]**	**4.14±2.47[bc]**
(*Z*)-3-Hexenyl acetate[d]	–	0.09±0.09	–	0.34±0.34	0.35±0.17	0.13±0.13
1-Octen-3-ol	**–[a]**	**0.29±0.29[a]**	**0.2±0.2[a]**	**0.12±0.12[a]**	**0.73±0.57[b]**	**0.66±0.21[b]**
Benzyl alcohol	0.31±0.05	–	0.14±0.07	0.4±0.21	0.17±0.09	0.27±0.14
Dodecane	0.31±0.06	0.56±0.21	0.30±0.08	0.63±0.11	0.33±0.08	0.41±0.1
Undecane	0.54±0.03	1.17±0.56	0.53±0.11	0.75±0.09	0.67±0.2	0.34±0.18
Octylaldehyde	0.27±0.05	0.19±0.19	0.08±0.08	0.28±0.28	0.1±0.1	0.19±0.12
Nonanal	0.68±0.34	1.33±0.66	0.75±0.17	0.96±0.96	0.38±0.38	0.86±0.54
Decanal	3.15±2.14	2.12±0.79	1.15±0.47	2.01±0.89	1.14±0.49	1.76±0.79
MeSA	**–[a]**	**–[a]**	**0.69±0.38[a]**	**2.48±1.62[a]**	**0.96±0.59[b]**	**2.75±1.36[b]**
Total	**6.45±2.69[a]**	**8.88±3.89[a]**	**9.29±3.94[a]**	**19.28±6.46[b]**	**33.12±17.79[b]**	**53.01±28.59[b]**

Bold letters represent the herbivore-induced compounds. Means in the same row followed by the same letters are not significantly different from each other ($P<0.05$). An asterisk (*) designates compounds which increased their emission with temperature ($P<0.05$)

[d] Compounds identified by comparison to authentic standards

Melitta, Minden, Germany) which is free of detectable amounts of volatiles (Kost and Heil 2005). The bags enclosed 4 stems of the target plant and were supported by a stainless steel rod on their outsides (Fig. 1). Compressed air (compressor: Schneider Druckluft GmbH, Reutlingen, Germany) entered the system after passing through an activated charcoal filter on the lower side of the plant and was pulled out at the top through an adsorbent super-Q filter by using a vacuum pump (Thomas Products, Memmingen, Germany) at the rate of 1.6±0.5 l min^{-1} for 30 min (Fig. 1). Before the initiation of volatile sampling, charcoal-filtered clean air was flushed though the collection system for 15 min; this was found to be sufficient in preliminary experiments to remove volatiles resulting from plant handling during the set-up of the enclosure. Umbrellas were employed to provide shade in order to moderate the temperature inside the bags on sunny days. Collections were made at the same time of day starting at 0900 h and continuing to 1500 h.

Throughout the volatile collection, temperature was measured inside each bag with temperature sensors; mean temperatures ranged from 20°C to 30°C. Radiation and temperature data also were recorded daily at the weather station at the "Jena Experiment" every 10 min. All temperature and light sensors were obtained from Campbell Scientific (Bremen, Germany).

To estimate the light reduction by the umbrellas, light under the umbrella was measured using a solar meter after the VOC sampling had been completed. Measurements were made continuously from 0900 to 1500 h for 3 consecutive days. The levels of radiation under the umbrella during the actual VOC sampling then were extrapolated by plotting a regression of measurements made under the umbrella to the weather station measurements on these 3 d. The equation

Table 2 Volatile emission from *Trifolium pratense* in the laboratory experiment (mean ± SE amount, ng leaf^{-1}h^{-1}) emitted after 4 hours of sampling at *30°C*

Compounds	Control	Mechanical Wounding	**Herbivory treatments**			
			30min	2h	8h	24h
α-Pinene	–	0.1±0.1	–	0.13±0.13	–	0.19±0.19
Sabinene	0.14±0.09	0.06±0.06	–	0.07±0.07	0.13±0.13	0.21±0.21
β-Pinene	–	0.15±0.09	–	0.09±0.09	0.16±0.16	0.21±0.12
Limonene*	0.67±0.3	0.44±0.14	0.31±0.15	0.43±0.14	0.52±0.34	0.29±0.15
1,8-Cineole	–	–	–	0.21±0.21	0.26±0.26	0.09±0.09
(*Z*)-β-Ocimene	**0.22±0.22^{a}**	**0.16±0.08^{a}**	**0.76±0.09^{ab}**	**3.42±3.02^{b}**	**6.17±3.14^{b}**	**5.63±1.5^{b}**
(*E*)-β-Ocimene	**1.15±0.69^{a}**	**0.66±0.26^{a}**	**3.66±0.19^{a}**	**15.57±13.86^{b}**	**26.55±11.05^{bc}**	**26.95±7.27^{c}**
Linalool	**0.3±0.3^{a}**	**–^{a}**	**0.22±0.12^{a}**	**0.16±0.16^{b}**	**0.16±0.09^{b}**	**0.29±0.06^{c}**
Indole	2.98±2.16	0.24±0.14	0.25±0.25	0.56±0.14	0.3±0.18	0.27±0.27
(*Z*)-Jasmone	0.21±0.1	0.15±0.07	0.25±0.13	0.13±0.13	0.51±0.51	0.62±0.54
(*E*)-β-Caryophyllene	**–^{a}**	**–^{a}**	**0.3±0.18^{b}**	**0.6±0.42^{b}**	**1.37±0.42^{b}**	**0.62±0.48^{b}**
(*E*)-β-Farnesene	0.3±0.3	0.1±0.1	–	0.16±0.16	0.47±0.47	0.3±0.15
Germacrene D	–	–	–	0.08±0.08	0.05±0.05	0.32±0.26
(*E,E*)-α-Farnesene*	**–^{a}**	**–^{a}**	**0.09±0.09^{b}**	**0.41±0.28^{b}**	**2.02±0.58^{c}**	**0.5±0.3^{b}**
DMNT	**–^{a}**	**0.09±0.09^{ab}**	**0.34±0.06^{ab}**	**0.17±0.17^{ab}**	**1.63±0.57^{bc}**	**1.18±0.31^{bc}**
(*Z*)-3-Hexenyl acetate	0.34±0.18	0.17±0.17	0.47±0.14	0.1±0.1	0.54±0.37	0.38±0.24
1-Octen-3-ol	**–^{a}**	**–^{a}**	**0.34±0.19^{a}**	**–^{a}**	**1.07±0.67^{b}**	**0.38±0^{b}**
Benzyl alcohol	0.52±0.37	0.09±0.09	0.12±0.12	–	0.09±0.09	0.08±0.08
Dodecane	0.88±0.44	0.43±0.12	0.48±0.19	0.58±0.13	0.51±0.11	0.29±0.16
Undecane	0.83±0.16	0.54±0.04	0.41±0.07	0.67±0.03	0.57±0.27	0.36±0.08
Octylaldehyde	0.35±0.17	0.19±0.19	0.2±0.1	0.22±0.11	0.3±0.09	0.15±0.07
Nonanal	2.37±0.9	1.19±0.32	1.44±0.4	1.46±0.06	1.48±0.45	1.15±0.26
Nonanal	2.37±0.9	1.19±0.32	1.44±0.4	1.46±0.06	1.48±0.45	1.15±0.26
Decanal	2.54±0.51	1.57±0.36	1.89±0.04	1.83±0.28	2.09±0.7	1.34±0.02
Decanal	2.54±0.51	1.57±0.36	1.89±0.04	1.83±0.28	2.09±0.7	1.34±0.02
MeSA	**0.12±0.12^{a}**	**0.49±0.49^{a}**	**0.48±0.25^{a}**	**0.39±0.39^{a}**	**1.86±0.17^{b}**	**1.04±0.61^{b}**
Total	**14.05±4.58^{a}**	**6.84±0.44^{a}**	**12.03±1.66^{a}**	**27.35±18.97^{b}**	**48.98±16.74^{b}**	**43.16±9.83^{b}**

Bold letters represent the herbivore-induced compounds. Means in the same row followed by the same letters are not significantly different from each other ($P<0.05$). An asterisk (*) designates compounds which increased their emission with temperature ($P<0.05$)

was: radiation under umbrella = 130.04+0.045 * radiation at weather station. For all analyses, the mean temperature and radiation during each collection interval was used.

Statistical Analysis

Emission data were log-transformed and analyzed using repeated measures analysis of variance with the open source software R 2.8.1 (http://www.r-project.org/; Crawley 2007). First, the interaction effects of herbivory treatments with temperature and sampling time were tested as main effects with plant as the random effect. The models were simplified to obtain the simplest models with highest explanatory power. Comparisons between feeding treatments were done after model simplification using factor level reduction.

For field data, the effect of radiation, temperature and herbivory were analyzed using a linear regression analysis. The emission data (ng g^{-1} h^{-1}) were square root transformed because this provided the best normality of errors and constancy of variance (Crawley 2007). DMNT, limonene, and myrcene occurred only a few times and were therefore converted to binomial data and analyzed using a generalized linear model.

For comparisons between the field and the laboratory experiments, proportions (percentages of total amounts) of 10 plant volatiles found to occur in both field and laboratory experiments were used. For the laboratory collections, only data from the 30 min sampling time were used, as it was similar to the sampling duration in the field study. After a *t*-test was performed to compare the composition of laboratory and field blends, a principal

Table 3 Volatile emissions of *Trifolium pratense* under field conditions (mean ± SE amount, ng $g^{-1}h^{-1}$) of compounds detected under field conditions. Means for 7 plots were calculated separately for two classes of herbivore damage, low and high

	Low damage	High damage
Monoterpenes		
α-Pinene[a]	2.78±0.76	2.05±1.36
Myrcene[a]	2.13±1.43	–
Limonene[a]	1.01±0.66	–
(*Z*)-β-Ocimene[a]	6.64±2.62	–
(*E*)-β-Ocimene[a]	23.42±6.50	5.57±2.91
Homoterpenes		
DMNT	1.77±0.95	–
Esters		
(*Z*)-3-Hexenyl acetate[a]	8.47±5.01	1.14±0.72
Hydrocarbons		
Unknown	10.05±2.17	15.97±4.14
Undecane	7.12±1.06	8.99 ±1.45
Dodecane	13.59±3.25	7.99 ±3.83
Aldehydes		
Decanal	15.06±7.30	31.34±6.56
Nonanal	11.74±1.91	3.97±2.81
Total	**103.79±20.52**	**77.02±16.11**

[a] Compounds identified by comparison with authentic standards

components analysis (PCA) was carried out to determine the correlations between low damage and high damage classes in the field and laboratory plants using CANOCO 4.5 (Ter Braak and Šmilauer 2002).

Results

VOC Emission by T. pratense Under Laboratory Conditions From headspace collections of *T. pratense* in the laboratory, 24 volatile compounds were identified by gas chromatography-mass spectrometry. The majority were terpenes: eight monoterpenes, four sequiterpenes and the homoterpene 4,8-dimethyl-1,3,7-nonatriene (DMNT). The remainder included eight aliphatic compounds and the aromatics, indole, methyl salicylate (MeSA) and benzyl alcohol (Tables 1, 2).

Effect of Herbivory Feeding by *S. littoralis* resulted in 2–30% damage on the experimental plants. This herbivory significantly increased both the total amount of volatiles released and the number of volatile compounds emitted (Tables 1, 2). A comparison across all treatments showed that herbivore feeding had a significant effect on emission of *(E)*-β-ocimene ($F_{5,30}$ = 9.09; P<0.01), *(Z)*-β-ocimene ($F_{5,30}$ = 4.52; P<0.01), DMNT ($F_{5,30}$ = 4.75; P<0.01), (*E,E*)-α-farnesene ($F_{5,30}$ = 4.00; P<0.01), (*E*)-β-caryophyllene ($F_{5,30}$ = 2.00; P<0.05), linalool ($F_{5,30}$ = 3.07; P<0.05) and MeSA ($F_{5,30}$ = 2.72; P<0.05) (Tables 1, 2). These seven compounds can, therefore, be considered herbivore-induced plant volatiles (HIPVs). There was no significant difference in emissions between mechanical wounding and control treatments for the six HIPVs based on factor level reduction (Tables 1, 2). The 30 min feeding treatment showed an increased emission in total volatiles from the control, but this increase was significantly different for only two specific compounds, (*E*)-β-caryophyllene and *(E)*-β-ocimene (P<0.05, Tables 1, 2). Plants exposed to 8 h and 24 h feeding emitted the highest amount of *(Z)*-β-ocimene, *(E)*-β-ocimene, and DMNT with no significant difference between the two feeding times. However, for (*E,E*)-α-farnesene, emission peaked at 8 h and then was lower after 24 h of feeding (Tables 1, 2). For the compounds *(Z)*-β-ocimene, MeSA and (*E*)-β-caryophyllene, the 2, 8, and 24 h feeding treatments did not differ significantly in emission (Tables 1, 2).

Effect of Temperature and Sampling Time Across all treatments, temperature had the strongest effect on emission of limonene ($F_{1,34}$ = 8.30; P<0.01). The amount of limonene emitted at 30°C was higher than that emitted at 20°C (P< 0.01, Fig. 2). Among the herbivore-induced volatiles, only (*E,E*)-α-farnesene emissions increased when temperature increased ($F_{1,24}$ = 5.93; P<0.05). There was an interaction between feeding treatment, temperature, and sampling time on the emission of (*E,E*)-α-farnesene ($F_{10,48}$ = 4.72; P< 0.01). Emission was higher after the 8 h feeding at 30°C and 4 h of sampling than after other treatments (P<0.01; Tables 1, 2).

Sampling time had an effect on the amounts of the terpenes α-pinene ($F_{2,70}$ = 4.75; P<0.05), β-pinene ($F_{9,70}$ = 9.33; P<0.01), 1,8-cineole ($F_{9,70}$ = 3.53; P<0.05), and (*Z*)-

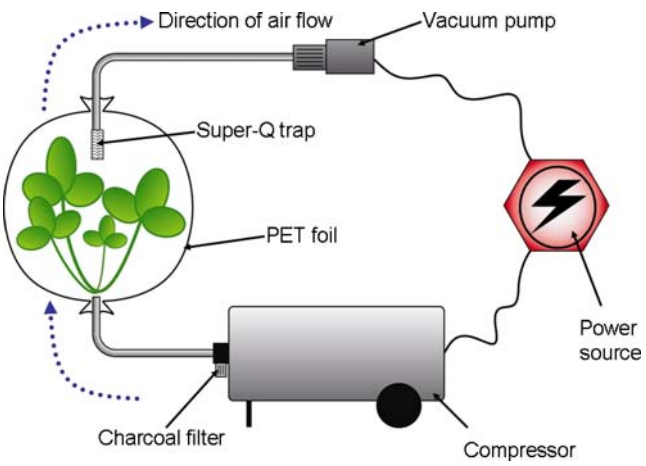

Fig. 1 Diagram of the VOC sampling system used in field collections

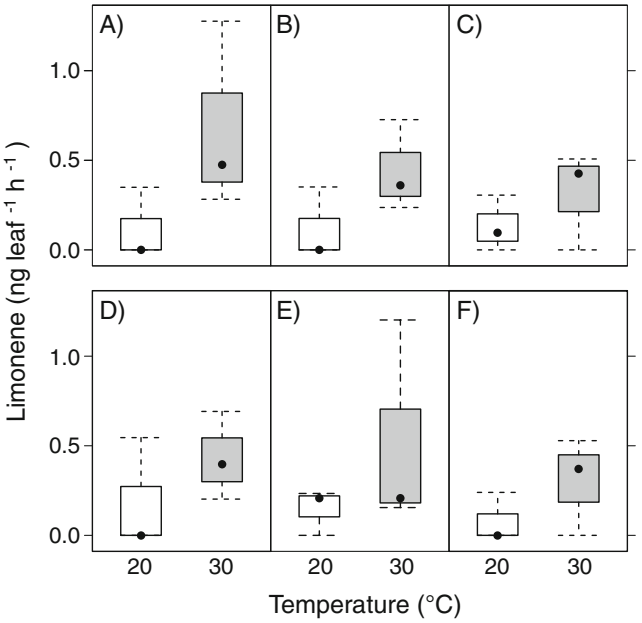

Fig. 2 Effect of temperature on the amount (ng leaf^{-1} h^{-1}) of limonene emitted from *Trifolium pratense* in the laboratory experiment. Panels show different treatments: (**A**) control, (**B**) mechanical wounding, (**C**) 30 min feeding, (**D**) 2 h feeding, (**E**) 8 h feeding, and (F) 24 h feeding. Dots in the boxes are the median values, while the bottom and top of the box show 25th and 75th percentiles respectively. Dashed vertical lines show either the maximum value or 1.5 times the interquartile range of the data (whichever is smaller). Open boxes depict data for 20°C and shaded boxes for 30°C

jasmone ($F_{9,70}$ = 8.35; P<0.01). These compounds were detected only after 4 h collection. Sampling time also had an interaction effect with feeding treatment on the emission of 1-octen-3-ol ($F_{10,60}$ = 2.08; P<0.05) where the effect of herbivory was detected after 4 h, but not after the 15 and 30 min sampling times. Thus, 1-octen-3-ol can also be regarded as an HIPV bringing the total number of such compounds in this study to eight.

The amounts of benzyl alcohol ($F_{2,70}$ = 3.00; P<0.05), (*Z*)-3-hexenyl acetate ($F_{2,70}$ = 47.4; P<0.01), decanal ($F_{2,70}$ = 4.75; P<0.05), dodecane ($F_{2,70}$ = 3.7; P<0.02) and undecane ($F_{2,68}$ = 86.95; P<0.01) all significantly decreased with increase in sampling time, while indole and octylaldehyde did not show variation with sampling time.

VOC Emission of T. pratense Under Field Conditions Under field conditions, eleven compounds were identified (Table 3), all of which were also detected in the laboratory. Levels of invertebrate herbivory measured as % leaf area loss ranged from 3–15%. Five compounds showed a significant response to herbivory in a multiple regression. The emission of α-pinene (T=4.06, P<0.01), *(Z)*-β-ocimene (T=5.21, P<0.01), *(E)*-β-ocimene (T=4.63, P<0.01), and nonanal (T=2.86, P<0.05) declined with increased herbivory (Table 3), while decanal increased (T=2.3, P=0.04), and undecane and dodecane were not significantly influenced by herbivory. The compound DMNT also was negatively correlated with herbivory but the correlation was not significant (T=2.1, P=0.6) There was no effect of herbivory on the emission of limonene and myrcene (T=1.9, P=0.2; T=1.6, P=0.2).

Effect of Temperature and Radiation Both radiation and temperature had significant effects on volatile emission in the field. An increase in radiation was negatively correlated with the emission of *(E)*-β-ocimene and nonanal (Fig. 3A, C), while α-pinene and decanal emissions appeared to increase with radiation,. although this increase was not significant (Fig. 3B, D). Similarly, undecane and dodecane emission were not significantly influenced by radiation (results not shown). Elevated temperature enhanced the emission of (*Z*)-3-hexenyl acetate (T=3.79, P<0.01), α-pinene (T=3.15, p=0.02), *(E)*-β-ocimene (T=3.05, P<0.05), and *(Z)*-β-ocimene (T=2.78, P<0.05). There were interactions between temperature and herbivory for *(E)*-β-ocimene (T=2.15, P<0.05) and between radiation and herbivory for α-pinene (T=4.06, P<0.05). *(E)*-β-Ocimene declined with an increase in herbivory at high temperature, while α-pinene emission decreased with herbivory at low radiation.

Comparison of Laboratory and Field Experiments Comparing field and laboratory data, herbivore-induced compounds usually were detected in lower relative amounts (% total compounds) in the field compared to the laboratory. The relative amounts of (Z)-β-ocimene and (E)-β-ocimene and limonene were lower in the field (T = −2.9, P=0.01; T = −3.374, P<0.01, and T = −3.45, P<0.01, respectively), while the amounts of α-pinene, decanal, and undecane were higher (T=3.35, P<0.01; T=2.09, P=0.05, and T=4.94, P<0.01, respectively). DMNT, nonanal, and (Z)-3-hexenyl acetate did not vary significantly in relative amount between field and laboratory conditions.

Principal components analysis of the relative amounts of volatiles emitted showed that the first axis (Principal factor 1) explained 60% of the variation in both field and laboratory experiments. The herbivore induced compounds, *(Z)*-β-ocimene and *(E)*-β-ocimene, were positively correlated with this axis, while decanal and undecane were negatively correlated with this axis (Fig. 4). In the laboratory, the more heavily damaged plants (>10% leaf damage) were positively correlated with herbivore-induced compounds (Fig. 4A), while in the field experiment, the less damaged plants (<10% leaf damage) were positively correlated with herbivore-induced compounds (Fig. 4B).

For (*E*)-β-ocimene, the dominant herbivore-induced compound in *T. pratense,* the proportion as a percentage of the total volatile blend generally increased with amount

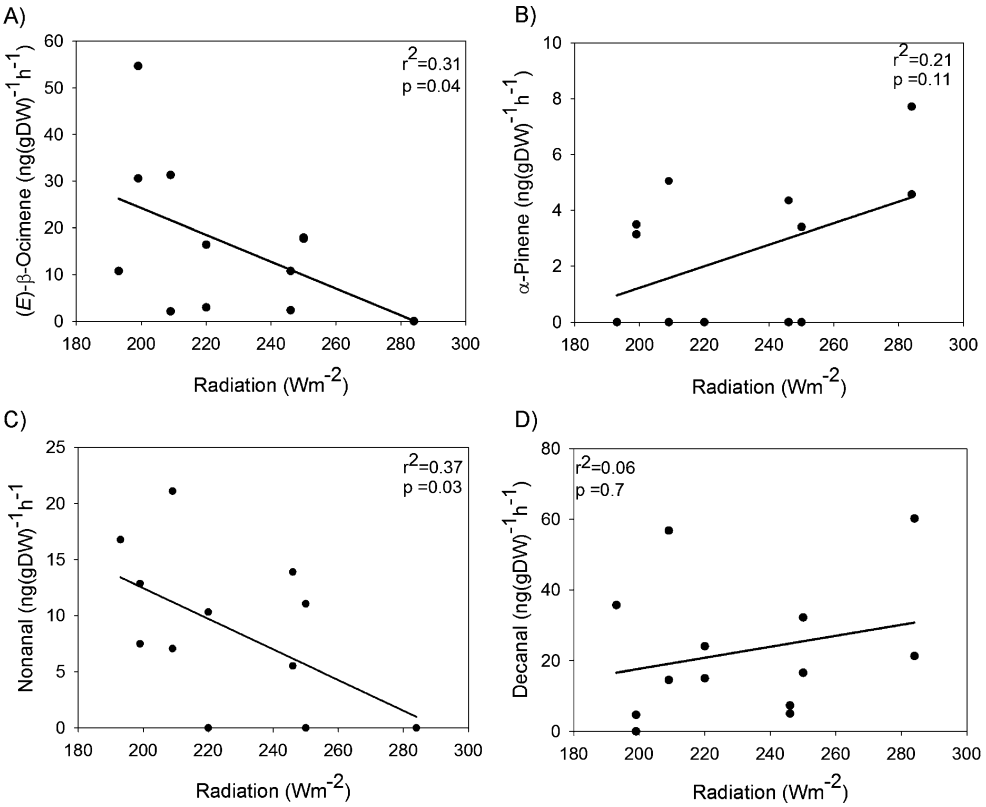

Fig. 3 Dependence of volatile emissions (ng (g DW)$^{-1}$h^{-1}) from *Trifolium pretense* on radiation measured in the field experiment. (**A**) (*E*)-β-ocimene (**B**) α-pinene, (**C**) nonanal, and (**D**) decanal

of damage under laboratory conditions (Fig. 5A), while in the field, intermediate levels of damage were associated with the greatest proportion of (*E*)-β-ocimene (Fig. 5B). To try and resolve this apparent contradiction, we reanalyzed the field emission data by removing the effects of radiation. (*E*)-β-Ocimene continued to show a decline with increased herbivory (Fig. 6A). Similarly, the emission of nonanal decreased with herbivory (Fig. 6C). However, amounts of α-pinene showed no trend in this regard (Fig. 6B), while the amounts of decanal increased with herbivory (Fig. 6D).

Discussion

Trifolium pratense is cultivated as forage and is found growing wild in a variety of grassland and ruderal habitats. Here, we report on the volatile compounds emitted by *T. pratense* growing under laboratory and field conditions. In the laboratory, feeding by the generalist lepidopteran *Spodoptera littoralis* significantly increased the amount of volatile organic compounds (VOCs) released, while elevated temperature did not significantly influence total emission. The release of eight individual compounds was herbivore-induced, of which the major compound, the monoterpene (*E*)-β-ocimene, made up 30–50% of the total. The other principal herbivore-induced plant volatiles (HIPVs) of this species were the isomeric monoterpene, (*Z*)-β-ocimene, the monoterpene linalool, the sesquiterpene (*E*)-β-caryophyllene, the sesquiterpene-derived homoterpene 4,8-dimethyl-1,3,7-nonatriene (DMNT) and methyl salicylate. Only three of these HIPVs were detected in the field. Surprisingly, their emission did not increase in relation to an increase in naturally-occurring levels of invertebrate herbivory, but rather decreased. Temperature and radiation significantly influenced the emission of all volatiles in the field except the aliphatic hydrocarbons.

The VOCs of *T. pratense* also have been reported from other plant species. DMNT, for example, has been detected frequently as an HIPV from plant species, such as maize, cotton, and cabbage. Such widespread HIPVs are suggested to be useful in attracting generalist natural enemies of herbivores (Turlings et al. 1998; De Boer and Dicke 2004). However, the low abundance HIPV, 1-octen-3-ol, so far has been documented from only a few plant species, including *Phaseolus lanatus* (lima bean) *Medicago truncatula*, and *Lotus japonicus* (Arimura et al. 2000; Ozawa et al. 2000; Leitner et al. 2005), all of which belong to the family Fabaceae. Interestingly, high amounts of 1-octen-3-ol also have been reported from mushrooms where they have anti-feeding effects on slugs (Wood et al. 2001). This compound could serve as an anti-feedant in *T. pratense* and other Fabaceae, but not much is known on the direct defense properties of HIPVs (Unsicker et al. 2009).

Among the volatiles of *T. pratense* are some that have been reported as HIPVs in other species, but were not induced in *T. pratense*. Indole, for example, was measured

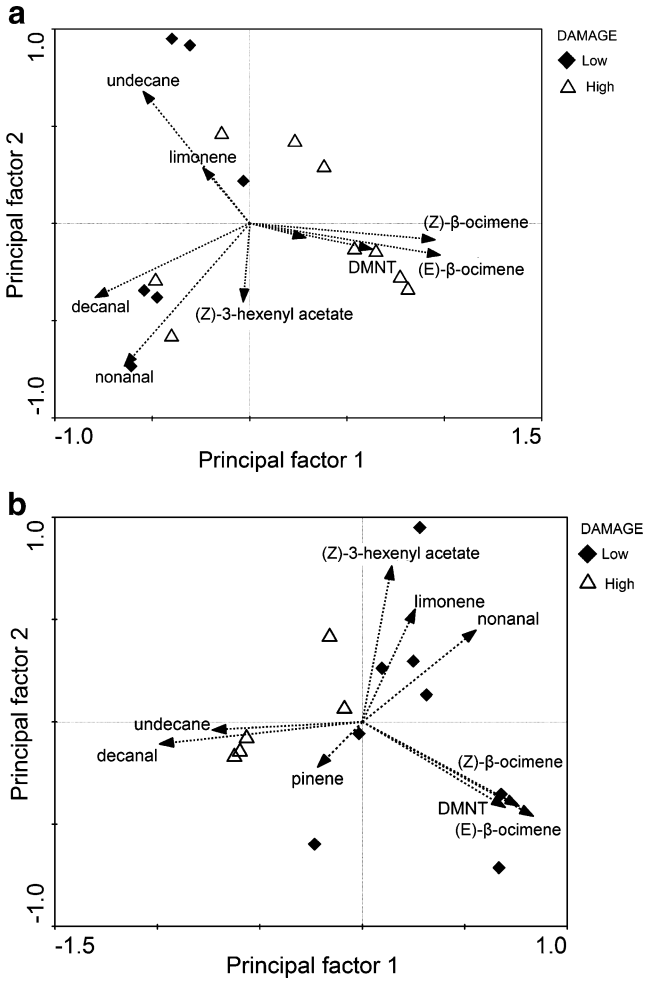

Fig. 4 Principal components analysis of the volatile emission of *Trifolium pratense* (**A**) in the laboratory experiment, and (**B**) in the field experiment based on the relative proportions of compounds in the headspace. Damage classes were allocated based on the percent damage to the leaf in both laboratory and field experiment. For the laboratory experiment, this ignored the effect of feeding duration. Damage was classified as high if it was more than 10%. Compounds are represented by arrows, each pointing in the direction of greatest increase. Smaller angles between arrows show higher correlation. Plant samples are indicated by symbols. Samples that cluster together are highly correlated, and samples and arrows that are in the same area are also correlated

only after herbivore attack in cotton and maize (Röse and Tumlinson 2004) but was constitutively emitted by *T. pratense*. (*Z*)-Jasmone also was not significantly induced in *T. pratense*, though it has been reported to be induced by feeding or by applying oral secretions in *Nicotiana attenuata*, maize, and cotton (Loughrin et al. 1994; Lou and Baldwin 2003; Röse and Tumlinson 2004). Another important feature of the HIPV composition of *T. pratense* is the dominance of (*E*)-β-ocimene (30–50%, Table 2). In other plant species, other HIPVs dominate the odor bouquet. For example, (*E*)-β-caryophyllene was the most dominant compound in several of the ecotypes of *Datura wrightii* in response to induction by methyl jasmonate or leaf beetle herbivory (Hare 2007), while *(Z)*-α-bergamotene was the main volatile compound in *Nicotiana attenuata* (Halitschke et al. 2000). Thus, volatile bouquets often are species specific. In the case of *T. pratense*, specificity may arise from the mixture of certain widespread volatiles, such as (*E*)-β-ocimene (*E*)-β-caryophyllene, DMNT, linalool, and MeSA with the Fabaceae-specific 1-octen-3-ol.

Herbivore-induced volatiles usually are reported to be first emitted 12–24 h after herbivory (Turlings et al. 1998; Heil 2008). However, a very rapid response to herbivory was exhibited by *T. pratense* in the laboratory where the HIPVs (*Z*)-β-ocimene, (*E*)-β- ocimene, DMNT, and MeSA were detected in the first 45 minutes after feeding was initiated (Table 2). This rapid release may be relevant to the proposed role of volatiles as within-plant or between-plant signals (Karban et al. 2000; Kessler et al. 2006; Heil and Silva Bueno 2007). To date, only green-leaf volatiles (GLVs) and MeSA have been shown to induce chemical defenses when applied to plants (Heil and Silva Bueno 2007; Park et al. 2007; Kost and Heil 2008), but the role of terpenes like *(E)*-β-ocimene needs further investigation in this regard (Gershenzon 2007). Besides the rapidly emitted compounds, *T. pratense* showed significant increases in the emission of some substances, including linalool, 1-octen-3-ol, (*E*)-β-caryophyllene, and (*E,E*)-α-farnesene, only after the plants were exposed to herbivory for more than 2 h. These compounds may be useful in attracting herbivore enemies, as has been shown for other species of plants, even though they are produced in relatively low amounts (less than 2 ng leaf^{-1} h^{-1}) (Table 2). Insects have been shown to detect relatively low amounts of VOCs, and higher emissions do not always correspond with increased response (Bruce et al. 2005).

There are only a few studies that directly compare herbivore-induced volatile production in the field and laboratory (Heil 2008; Dicke 2009). In our work, many compounds identified in the laboratory, such as indole, were not detected in the field. This discrepancy could be due to differences in growth conditions. Indole, for example, is often produced as a result of physiological stress (Dudareva et al. 2004). This compound may have been produced in the growth chamber used for the laboratory experiments, since many climate chambers are known to be oxygen-rich. Other compounds identified in laboratory collections, such as sabinene, 1,8-cineole, β-pinene, germacrene D, (*Z*)-jasmone (*E,E*)-α-farnesene, and (*E*)-β-farnesene, were not detected possibly due to the shorter collection time of 30 min used in the field. These compounds also were not detected under laboratory conditions within a 30 min collection. A short collection time was deemed necessary in the field to minimize physical stress on plants and to allow sampling of many plants.

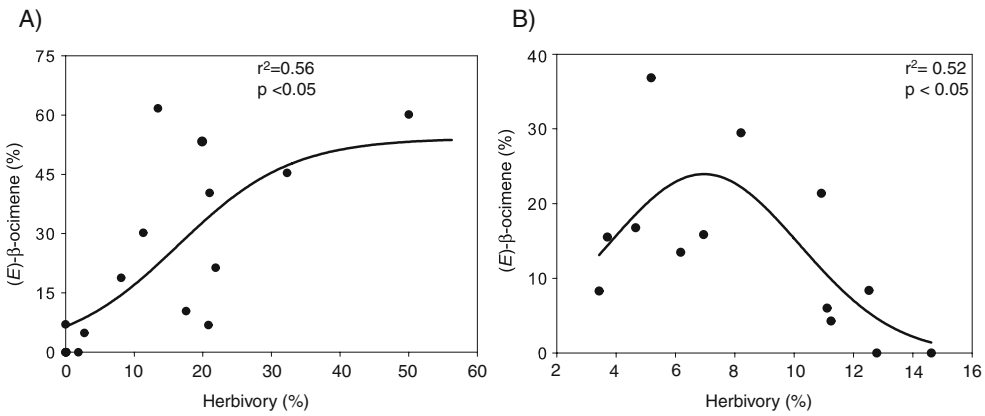

Fig. 5 Effect of herbivory on emission of (*E*)-β-ocimene (expressed as percentage of total blend) from *Trifolium pratense* under (**A**) laboratory and (**B**) field conditions

The differences in volatile composition observed between laboratory and field collections also might be attributed to differences in the apparatus used. Plants were enclosed in glass jars in the lab and in polyethylene terephthalate (PET) bags in the field. However, volatile recovery rates between glass vials and PET bags should not differ. Comparison of the recovery rates of VOCs from mixtures of standard compounds in glass vessels vs. PET bags showed no significant differences for limonene, linalool, MeSA, and (*E*)-β-caryophyllene (Stewart-Jones and Poppy 2006). Ambient air may contain ozone, which breaks down many volatile compounds Pinto et al. (2007). Contamination of the airflow in the PET bags was avoided by using over-pressure push-pull system (Fig. 1).

The main cause of differences between field and laboratory volatile collections thus is likely to be differences in the environmental conditions, such as light, atmospheric composition, and water supply that plants were growing under, although it is difficult to identify specific factors without further investigation. A similar reduction in the number of volatile compounds detected under field conditions compared to controlled laboratory

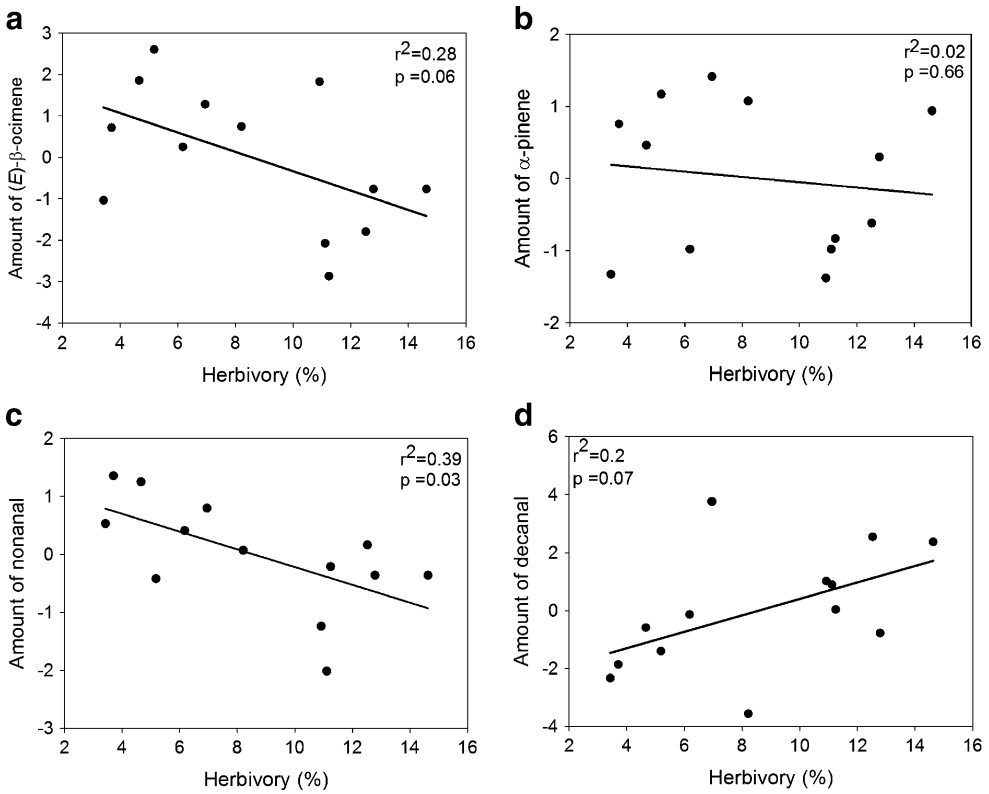

Fig. 6 Dependence of volatile emissions (ng (g DW)$^{-1}$h^{-1}) from *Trifolium pretense* on measured herbivory rates in the field experiment. (**A**) (*E*)-β-ocimene, (**B**) α-pinene, (**C**) nonanal, and (**D**) decanal after removing the effect of radiation. Amount of each compound is expressed as a residual

conditions also has been reported by Tooker et al. (2008) for *Solidago altissima*.

High levels of damage in the field were unexpectedly negatively correlated with herbivore-induced volatiles, unlike in the laboratory where damage led to an increase in volatile emission with eventual saturation (Table 3, Figs. 4 and 5). The plants selected for volatile collection in the field already had been damaged by herbivores at unknown time points prior to sampling, while in the laboratory induced volatile measurements were all performed within 24 h after herbivory was stopped. Laboratory measurements thus represent a snapshot taken right at the beginning of the time course of volatile emission following herbivory, while measurements in the field may have been made many days after herbivory.

The negative correlation of herbivory to volatile emission in the field (Fig. 4) also may be rationalized in several other ways. First, field plants that are attacked by herbivores may not allocate resources directly to defensive compounds, but to regrowth capabilities instead. *T. pratense* is a perennial plant that often behaves like a biennial with the ability to re-grow rapidly (Taylor and Quesenberry 1996). High herbivory may have induced a re-translocation of carbon and nitrogen to the roots in order to allow better re-growth in the later phases of development (Schwachtje and Baldwin 2008). Alternatively, resources that could have been invested in volatile formation instead may have been allocated to the formation of other chemical defenses. *T. pratense* has been shown to produce other secondary metabolites like clovamides in response to diseases or herbivory (Macfoy and Smith 1979; McMurchy and Higgins 1984; Tebayashi et al. 2000). Analysis of other induced defense compounds would provide more information on how this species responds in nature to herbivory.

Second, herbivory in the field may not be positively correlated to herbivory in the laboratory because of differences in the species of herbivore causing damage in the field as well as to the presence of pathogens. Different herbivores are known to induce different volatiles due to differences in elicitors present in their saliva (Turlings et al. 1993;Geervliet et al. 1997; De Moraes et al. 1998). For example, Geervliet et al. (1997) reported both qualitative and quantitative differences in the volatiles emitted by plants when fed on by two different caterpillar species. At the time of sampling in our study, the main observable herbivores on *T. pratense* were beetles and grasshoppers. Grasshoppers like the generalist species *Chorthippus parallelus* have also been shown to feed on *Trifolium pratense* in food choice experiments (Unsicker et al. 2008; Franzke et al. 2009). Grasshoppers contain different elicitors from *S. littoralis* (Alborn et al. 1997, 2007) that result in differences in the volatiles emitted by plants (Turlings et al. 1993). Since herbivory in the laboratory was induced with *S littoralis*, the differences in herbivores in the field may have caused the differences in quality and quantity of the volatile blend. In addition, beetle larvae, which have been reported to feed on *T. pratense* roots (Tapia et al. 2007), may have affected volatile emission. Thus it is possible that the plant measured may have had beetle larvae feeding on the roots. Such multiple agents of herbivory, plus pathogen infestations, which were also observed in the field, may have altered and even suppressed VOC production (Maddox and Root 1990; Tooker et al. 2008).

A third reason why herbivory in the field might have been associated with lower volatile emission is that a lower level of emission may have resulted in fewer visits from natural enemies and thus a higher rate of herbivory (Halitschke et al. 2000; Heil 2008). Finally, our experimental plants in the field grew together with other species (Appendix 1), which may have influenced volatiles, although it is still unclear how species composition of communities affects volatile emission. However, other induced defense compounds are affected by neighboring species mainly through competitive interactions (Van Dam and Baldwin 2001; Marak et al. 2003; Barton and Bowers 2006). For example, Barton and Bowers (2006) reported an increase in iridoid glycosides in the presence of competitors, and this increase was dependent on neighbor identity.

The influence of temperature and radiation on the emission of specific volatiles was consistent with trends previously reported in the literature with elevated temperature and radiation generally increasing emission (Gouinguene and Turlings 2002; Kuhn et al. 2002; Blanch et al. 2007). Monoterpenes are especially light and temperature sensitive (Guenther et al. 1993; Kesselmeier and Staudt 1999). In the laboratory experiment, limonene, and the herbivore-induced compound (*E,E*)-α-farnesene exhibited increased emission upon raising the temperature from 20°C to 30°C (Tables 1, 2, Fig 2). In the field, increased temperature was correlated with increased emission of (*Z*)-3-hexenyl acetate, and the terpenes, (*E*)-β-ocimene, (*Z*)-β-ocimene, and α-pinene, with the remainder of compounds showing no significant increases or decreases with temperature. Meanwhile, increased radiation in the field apparently correlated with increased α-pinene and decanal but was not statistically significant, (Fig. 3). Counter to these trends, is the unexpected decline in the emission of the principal volatiles, (*E*)-β-ocimene and nonanal with increased radiation. This may have been due to the use of umbrellas for field collection on the warmer, sunny days. The resulting lower radiation on such days would be associated with higher temperatures, with the net result being a positive correlation between lower radiation and higher emission.

In summary, our results emphasize the need for caution in extrapolating the volatile emission profiles observed in laboratory experiments to plants growing in the field. Many of the compounds measured in the laboratory were not

detected in the field, and many compounds observed to be induced by herbivory in the laboratory were not positively associated with herbivore damage in the field. The changes in light and temperature conditions between the lab and field could not account for these differences. Perhaps other abiotic factors not measured in this study were responsible for the variation, such as atmospheric gas composition, light quality, or water supply. Or, perhaps the combined influence of many biotic factors in the field, including multiple herbivores (both shoot and root), pathogens, and competing plants shape a vastly different emission profile. The damage observed in the field may have been too old to have an influence on volatile emission.

Future studies need to take into account biotic and abiotic factors that may influence emission in the field especially those, such as the presence of multiple herbivores and competing plants, which cannot be easily simulated in the laboratory. Such work preferably should manipulate herbivory directly on field-grown plants and use this as a basis to make comparisons with emission from plants with previously existing herbivore damage. More complete and accurate knowledge of the range of volatiles emitted from field plants is essential if we are to make progress in understanding the physiological and ecological functions of these compounds.

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are implicated in a large number of physiological functions, such as plant pollination (Raguso 2008), attraction of seed dispersers (Borges et al. 2008), and defense against biotic and abiotic stresses (Pichersky and Gershenzon 2002; Vickers et al. 2009). VOCs may have a direct deterrent or cytotoxic effect against herbivores and pathogens (De Moraes et al. 2001; Pichersky and Gershenzon 2002; Heil 2004), or indirect effects through the attraction of herbivore enemies as predators or parasitoids (Turlings and Wäckers 2004; Heil 2008). The defensive function of VOCs has been studied intensively in some model plant species (Turlings and Wäckers 2004) but not much is known about importance in complex plant communities such as tropical forests.

Some compounds are common in both reproductive and vegetative plant tissues. In a review on floral scents, Knudsen et al. (2006) emphasized the importance of twelve compounds that occur in over 50% of the 90 plant families investigated. They highlighted six monoterpenes (D-limonene, (E)-β-ocimene, β-myrcene, linalool, α-pinene, β-pinene), one irregular terpene (6-methyl-5-hepten-2-one), four shikimic acid pathway by-products (benzaldehyde, methyl-2-hydroxybenzoate, benzyl alcohol, 2-phenyl ethanol), and one sesquiterpene (β-caryophyllene). Aside from their likely role in plant pollination, most of these have toxic or deterrent activity against microbes and herbivores (De Moraes et al. 2001; Mumm and Hilker 2006; Bakkali et al. 2008). It has been proposed that the widespread distribution of these compounds and their emission by both reproductive and vegetative parts of the plant reflect their importance in chemical defense (Knudsen et al. 2006). We expect that they also will be widespread among tropical tree species, as a response to high predation pressure.

Aside from the common compounds, one of the striking characteristics of plant volatile terpenes is their diversity and the large number of compounds in blends (Gershenzon and Dudareva 2007). The "screening hypothesis" proposes that the ability to produce a large array of defensive compounds increases the probability of possessing active compounds (Jones and Firn 1991; Firn and Jones 2003). A high chemical diversity should help increase the plant's protection against attacks from a wide range of enemies in changing environments (Agrawal and Fishbein 2006; Gershenzon and Dudareva 2007). Moreover, diverse compounds may act synergistically to provide greater toxicity or deterrence (Berenbaum and Neal 1985), or to maintain activity for a longer period of time (Akhtar and Isman 2003). Complex VOC blends also may be involved in making the information sent to parasitoids in tritrophic interactions species-specific (Gershenzon and Dudareva 2007). Thus, the diversity of VOCs in a species may be at least in part related to defensive roles. Studying patterns of VOC diversity and variability across species may shed light on their importance in long-lived species such as tropical trees.

Both the density and diversity of insects are higher in the tropics than in temperate zones (Novotny et al. 2006; Lewinsohn and Roslin 2008). This leads to higher predation pressure on tropical plants compared with their temperate counterparts (Janzen 1970; Coley and Aide 1991). Consequently, tropical plants may have enhanced defenses compared to temperate plants, including more diversity and variability in chemical defenses (Coley and Aide 1991). We postulated that many different VOCs would be released by tropical tree species, reflecting a diverse array of predatory pressure. Moreover, the use of VOCs in defense may not be uniform among species, and this should lead to mixtures of varying complexities.

We sampled the vegetative parts (bark and leaves) of a wide array of tropical plants in natural forests in French Guiana (South America), and identified the blend of constitutive VOCs released immediately in response to physical damage. We used this dataset to determine patterns of VOC diversity among tropical tree species with three objectives: (1) What is the relative frequency of VOCs across species? (2) Are mixtures of VOCs species specific? (3) Is the complexity of the mixtures variable across species?

Methods and Materials

Study Sites Field work was conducted at three sites of old-growth rainforest in French Guiana, namely the Paracou Research Station (5°18′N, 52°53′W), the Nouragues Research Station (4°05′N, 52°40′W), and Montagne Tortue (4°18′N, 52°22′W). Annual rainfall is 2,990 mm for Nouragues, 3,160 mm for Paracou, and ca. 3,500 mm for Montagne Tortue. We worked on five plots of one hectare each: two were at the Paracou site (N=643 and 487 trees), two at Nouragues (N=537 and 567 trees), and one at Montagne Tortue (N = 536 trees). In these plots, each tree with a diameter at breast height (dbh) greater than 10 cm was climbed by professional tree climbers (Baraloto et al. 2009) to collect leaves in order to create a voucher specimen for taxonomic determination. Over 98% of the trees were identified to species or morphospecies, and the species richness ranged from 148 to 210 species per hectare. Sampling was conducted in June 2007 for Paracou, September 2007 for Nouragues, and in November 2007 for Montagne Tortue.

Field Sampling We sampled species belonging to the most common tree families across sites and for which our plots had more than one individual per species. Overall, 2–6 individuals of the most common species were chosen,

depending on the individuals available, totaling 195 individuals from 55 species. Table 1 lists the sampled species and sampling size. Sampling a large number of species requires a rapid technique and appropriate storage for subsequent analysis (Wajs et al. 2006). For each tree, we cut about 20 mg of tissue from a young leaf. Young leaves were chosen because they are better chemically defended than older ones (McKey 1979). We also sampled a 1-cm^2 piece of bark at 1 m above ground, using a leather punch. Each sample was put immediately into a glass vial (10 ml) and sealed with a screw cap containing a Teflon-lined septum (Varian Instruments, Sunnyvale, CA, USA). In the field, sealed vials were maintained at −4°C in a portable freezer. Samples were transferred to the laboratory where they were stored at −20°C until analysis.

By using four taxa representative of our dataset (*Protium sp.* Burseraceae, *Inga sp.* Mimosaceae, *Guarea sp*. Meliaceae, *Spondias mombin* Anacardiaceae), we verified that the VOC composition as measured with our protocol was consistent with the blend released following mechanical damage of the tissue (either bark or leaves). For 3 individuals per species, we trapped VOCs directly in the field by sealing mechanically damaged tissue in a Teflon bag, and then introducing a Solid Phase Micro Extraction (SPME) fiber into the bag for 5 to 15 min (Bouvier-Brown et al. 2007). For the same tissue, we collected VOCs from frozen samples as described below.

Laboratory Analyses VOCs from the headspace of the samples were adsorbed onto SPME fibers, allowing analysis without solvent extraction (Lord and Pawliszyn 2000). Originally developed for the analysis of pollutants, this technique is suitable for detecting VOCs emitted by plants (Tholl et al. 2006; Mayer et al. 2008). We used fused silica fibers coated with PDMS/DVB (Polydimethylsiloxane/Divinylbenzene) 65 µm (Supelco, Bellefonte, PA, USA), since this coating is an effective trap for plant VOCs (Guo et al. 2006; Bouvier-Brown et al. 2007). Fibers were conditioned before the first use for 30 min at 250°C, following instructions from the manufacturer. Before extraction, the glass vials containing the tissue samples were maintained at room temperature for at least 1 h. The SPME fiber was placed into the vial with the tissue sample (bark or leaf) for 5 to 60 min at ambient temperature (25° C). The exposure time was optimized for each species to maximize the extraction without saturating the analytical column (Appendix 1). The fiber was inserted immediately into the 250°C inlet of a Varian 3800 Gas Chromatograph (GC) fitted with a Saturn 2000 ion-trap Mass Spectrometer (MS; Varian Instruments, Sunnyvale, CA, USA). The GC was run with a non-polar Varian DB-5 column (30 m× 0.25 mm ID, 0.25 µm film) commonly used for the analysis of VOCs (Tholl et al. 2006). Helium was the carrier gas at a constant flow of 1 ml/min. The oven temperature program of the GC started at 50°C, with 6°C/min temperature increase up to 140°C, and then with 4°C/min increments up to 160°C. This temperature was held for 1 min and increased finally to 200°C at 10°C/min. The MS was operated in electronimpact (EI) mode at 70 eV, with a scan range of 30–450 m/z. After each analysis, the fibers were cleaned in the injector port for 10 min at 250°C, and each fiber was reused no more than 100 times, as recommended by Tholl et al. (2006). Control analyses (blanks) were performed every ten analyses to check for contamination of the fiber.

Post-Processing of the GC-MS data Since the SPME technique does not ensure quantitative recovery, only presence/absence data are reported. VOC presence/absence was inferred by using a novel statistical approach (Nicole et al., unpublished). This procedure is both more efficient and more accurate than visual classification procedures for a large number of analyses. All routines were developed in the R statistical software (http://cran.r-project.org/). Here, we review briefly the major steps in the procedure.

A typical GC-MS output is represented by two components. The chromatogram displays the mixture as separated by the GC, and each peak corresponds to the elution of a distinct molecule, characterized by retention time. For each point on the GC chromatogram (N=1,500), a mass spectrum is obtained by fragmentation in the MS chamber. A mass spectrum is represented by a histogram displaying the intensity of each fragment (the mass-to-charge ratio, m/z).

The GC-MS output was saved into a raw text file by using the *GC and GCMS File Translator*™ (ChemSW, http://www.chemsw.com/12149.htm). Peaks were identified automatically in each chromatogram as a 3-point increase followed by a 3-point decrease around a peak intensity of at least 5 kcounts. Each detected peak in the course of the analysis was then defined by the mass spectrum that corresponded to the apex of the peak. The mass spectra corresponding to all peaks in all analysis were grouped into a single matrix in which each row represented the mass spectrum associated with a selected GC peak. A distance matrix was computed by using the pairwise Euclidian distance between any two spectra. An agglomerative clustering algorithm was used to cluster the spectra based on this distance matrix (Ward 1963), and the optimal number of clusters was inferred from a variance analysis (Rousseeuw 1987).

The Kováts Retention Index (RI, Kovats 1958) for each peak were defined as follows

$$RI(i) = 100 \times \left(\frac{\ln(RT(i)) - \ln(RT_{\inf}(i))}{\ln(RT_{\sup}(i)) - \ln(RT_{\inf}(i))} \right) + 100n$$

Table 1 Number of individuals analyzed sorted by order, family, genus, and species

Order	Family	Genus	Species	Species code	#
Magnoliales	Annonaceae	*Duguetia*	*surinamensis*	*D. surinamensis*	3
Magnoliales	Annonaceae	*Oxandra*	*asbeckii*	*O. asbeckii*	4
Magnoliales	Annonaceae	*Unonopsis*	*perrottetii*	*U. perrottetii*	5
Magnoliales	Annonaceae	*Unonopsis*	*rufescens*	*U. rufescens*	3
Magnoliales	Annonaceae	*Xylopia*	*nitida*	*X. nitida*	4
Magnoliales	Myristicaceae	*Iryanthera*	*hostmannii*	*I. hostmannii*	2
Magnoliales	Myristicaceae	*Iryanthera*	*sagotiana*	*I. sagotiana*	2
Magnoliales	Myristicaceae	*Virola*	*michelii*	*V. michelii*	2
Laurales	Lauraceae	*Aniba*	*panurensis*	*A. panurensis*	2
Laurales	Lauraceae	*Ocotea*	*argyrophylla*	*O. argyrophylla*	2
Laurales	Lauraceae	*Ocotea*	*percurrens*	*O. percurrens*	2
Laurales	Lauraceae	*Sextonia*	*rubra*	*S. rubra*	4
Myrtales	Myrtaceae	*Myrcia*	*decorticans*	*M. decorticans*	3
Myrtales	Vochysiaceae	*Ruizterania*	*albiflora*	*R. albiflora*	3
Malpighiales	Caryocaraceae	*Caryocar*	*glabrum*	*C. glabrum*	3
Malpighiales	Chrysobalanaceae	*Hirtella*	*glandulosa*	*H. glandulosa*	4
Malpighiales	Chrysobalanaceae	*Licania*	*membranacea*	*L. membranacea*	5
Malpighiales	Chrysobalanaceae	*Parinari*	*campestris*	*P. campestris*	2
Malpighiales	Clusiaceae	*Rheedia*	*madruno*	*R. madruno*	3
Malpighiales	Clusiaceae	*Tovomita*	*spB1*	*T. spB1*	3
Malpighiales	Euphorbiaceae	*Conceveiba*	*guianensis*	*C. guianensis*	5
Fabales	Caesalpiniaceae	*Tachigali*	*melinonii*	*T. melinonii*	2
Fabales	Caesalpiniaceae	*Vouacapoua*	*americana*	*V. americana*	3
Fabales	Papilionaceae	*Bocoa*	*prouacensis*	*B. prouacensis*	3
Rosales	Cecropiaceae	*Pourouma*	*villosa*	*P. villosa*	3
Rosales	Moraceae	*Brosimum*	*guianense*	*B. guianense*	6
Sapindales	Anacardiaceae	*Anacardium*	*spruceanum*	*A. spruceanum*	5
Sapindales	Anacardiaceae	*Thyrsodium*	*guianense*	*T. guianense*	4
Sapindales	Anacardiaceae	*Thyrsodium*	*puberulum*	*T. puberulum*	4
Sapindales	Burseraceae	*Dacryodes*	*nitens*	*D. nitens*	4
Sapindales	Burseraceae	*Protium*	*decandrum*	*P. decandrum*	5
Sapindales	Burseraceae	*Protium*	*opacum*	*P. opacum*	4
Sapindales	Burseraceae	*Protium*	*sagotianum*	*P. sagotianum*	4
Sapindales	Burseraceae	*Tetragastris*	*altissima*	*T. altissima*	4
Sapindales	Burseraceae	*Tetragastris*	*panamensis*	*T. panamensis*	4
Sapindales	Meliaceae	*Carapa*	*procera*	*C. procera*	3
Sapindales	Sapindaceae	*Cupania*	*scrobiculata*	*C. scrobiculata*	5
Sapindales	Simaroubaceae	*Simaba*	*cedron*	*S. cedron*	5
Malvales	Bombacaceae	*Pachira*	*dolichocalyx*	*P. dolichocalyx*	3
Malvales	Sterculiaceae	*Sterculia*	*pruriens*	*S. pruriens*	4
Malvales	Sterculiaceae	*Theobroma*	*subincanum*	*T. subincanum*	4
Malvales	Tiliaceae	*Apeiba*	*glabra*	*A. glabra*	4
Ericales	Lecythidaceae	*Eschweilera*	*congestiflora*	*E. congestiflora*	3
Ericales	Lecythidaceae	*Eschweilera*	*coriacea*	*E. coriacea*	3
Ericales	Lecythidaceae	*Lecythis*	*persistens*	*L. persistens*	4
Ericales	Lecythidaceae	*Lecythis*	*poiteaui*	*L. poiteaui*	4
Ericales	Sapotaceae	*Chrysophyllum*	*argenteum*	*C. argenteum*	3
Ericales	Sapotaceae	*Micropholis*	*egensis*	*M. egensis*	3
Ericales	Sapotaceae	*Micropholis*	*guyanensis*	*M. guyanensis*	5

Table 1 (continued)

Order	Family	Genus	Species	Species code	#
Ericales	Sapotaceae	*Pouteria*	*gonggrijpii*	*P. gonggrijpii*	3
near Gentianales	Icacinaceae	*Poraqueiba*	*guianensis*	*P. guianensis*	4
Gentianales	Apocynaceae	*Aspidosperma*	*cruentum*	*A. cruentum*	3
Gentianales	Apocynaceae	*Aspidosperma*	*marcgravianum*	*A. marcgravianum*	4
Gentianales	Rubiaceae	*Chimarrhis*	*turbinata*	*C. turbinata*	4
Gentianales	Rubiaceae	*Posoqueria*	*latifolia*	*P. latifolia*	3
					195

where $RT(i)$ is the retention time of the i^{th} peak (in minutes), $RT_{inf}(i)$ is the retention time of a reference n-alkane that eluted immediately before the i^{th} peak, and $RT_{sup}(i)$, the retention time of (n+1)-alkane that eluted immediately after the i^{th} peak. In the above equation, n is the number of carbon atoms in the n-alkane that eluted immediately before the i^{th} peak. Together with the mass spectrum, RI is used for the identification. The consistency of the clusters generated automatically was tested by computing the intra-cluster variance of RI. Ambiguous clusters were examined separately and found to correspond either to very similar compounds that can be distinguished with RI or to several co-eluted compounds. Clusters were identified based on comparison to authentic mass spectral standards or to the NIST 98 MS library, the ADAMS library, and to RI reported in the literature (ADAMS). Using these techniques we were able to assign 78% of the compounds as known molecular structures (Appendix 2). For unidentified molecules, we defined them as unknown molecules characterized by their mass spectra and their RI.

Validation of the Protocol We verified our methods by comparing the VOCs obtained from SPME fibers exposed to freshly harvested tissue in the field to those obtained with our lab techniques that used frozen tissue. The qualitative blend of VOCs was the same with the two methods for four different species tested (Fig. 1). During the period of analysis, we confirmed that storage of tissue samples at −20°C did not alter VOC composition by repeating the analyses on three samples at monthly intervals (data not shown). We concluded that the methods were an effective way to sample VOCs from the large number of samples collected in the field study.

Statistical Analyses For each individual tree, the VOC composition was defined as the composite of all compounds found in the leaves, in the bark or in both tissues. The resulting dataset was characterized by a presence/absence matrix, where each row represented the compounds found in an individual tree, and each column represented a single compound. We calculated the chemical richness (number of distinct compounds) for each individual, each species, and each plant order. The significance of differences in mean VOC diversity (the number of compounds)

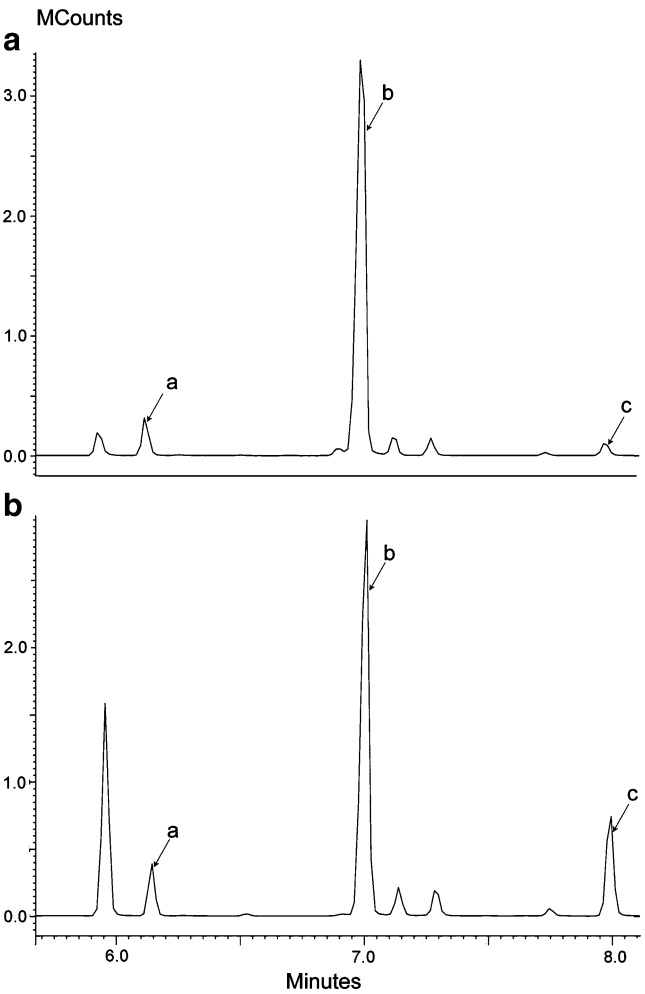

Fig. 1 Comparison of the composition in monoterpenes for the leaves of one individual of *Protium sp.*, for **a** extraction in the field and **b** extraction in glass vials from frozen samples. Similar results were obtained for the other species. Compounds shown are: **a** α-pinene, **b** sabinene, **c** γ-terpinene

among species and orders were tested with the non parametric *Kruskal-Wallis* test.

To assess whether our dataset could be used to discriminate among species, we constructed a matrix of chemical dissimilarity between pairs of individuals using the Manhattan distance $D_{x,y} = \sum_{i=1}^{N_m} |x_i - y_i|$, where x and y are two distinct individuals, and N_m is the total number of compounds in our dataset. We then constructed a hierarchical dendrogram with the Ward clustering algorithm (Ward 1963). Dendrogam node support was assessed based on approximately unbiased (AU) p-values computed from 10,000 bootstraps (pvclust package; Suzuki and Shimodaira 2006).

Results

A total of 264 compounds was found in the 55 sampled species. This included one nitrogen-containing compound, three compounds from the shikimic acid pathway, 34 compounds from the lipoxygenase pathway (or green leaf volatiles), 57 monoterpenes, and 169 sesquiterpenes. We were able to assign names to 206 of the 264 compounds (78%). The 58 unidentified compounds are mostly sesquiterpenes, and many of them may be previously unreported.

Four terpenes were released by over 90% of the species (Fig. 2): α-pinene (all species) and D-limonene (96% of the species) in the monoterpene group, and β-caryophyllene (94% of species) and α-copaene (92% of species) in the sesquiterpene group. About 23% of the compounds were present in the VOC blend of only a single species (Fig. 2).

For 43 of the 55 sampled species, the clustering analysis grouped all individuals of the same species in well-supported clusters (AU *P*-value greater than 0.80; Fig. 3). Above the species level, this clustering analysis did not show any pattern of chemical similarities within genera or families (Fig. 3). Similar results were obtained by considering only monoterpenes and sesquiterpenes.

The complexity of the VOC profile varied significantly both across all species and within each plant order

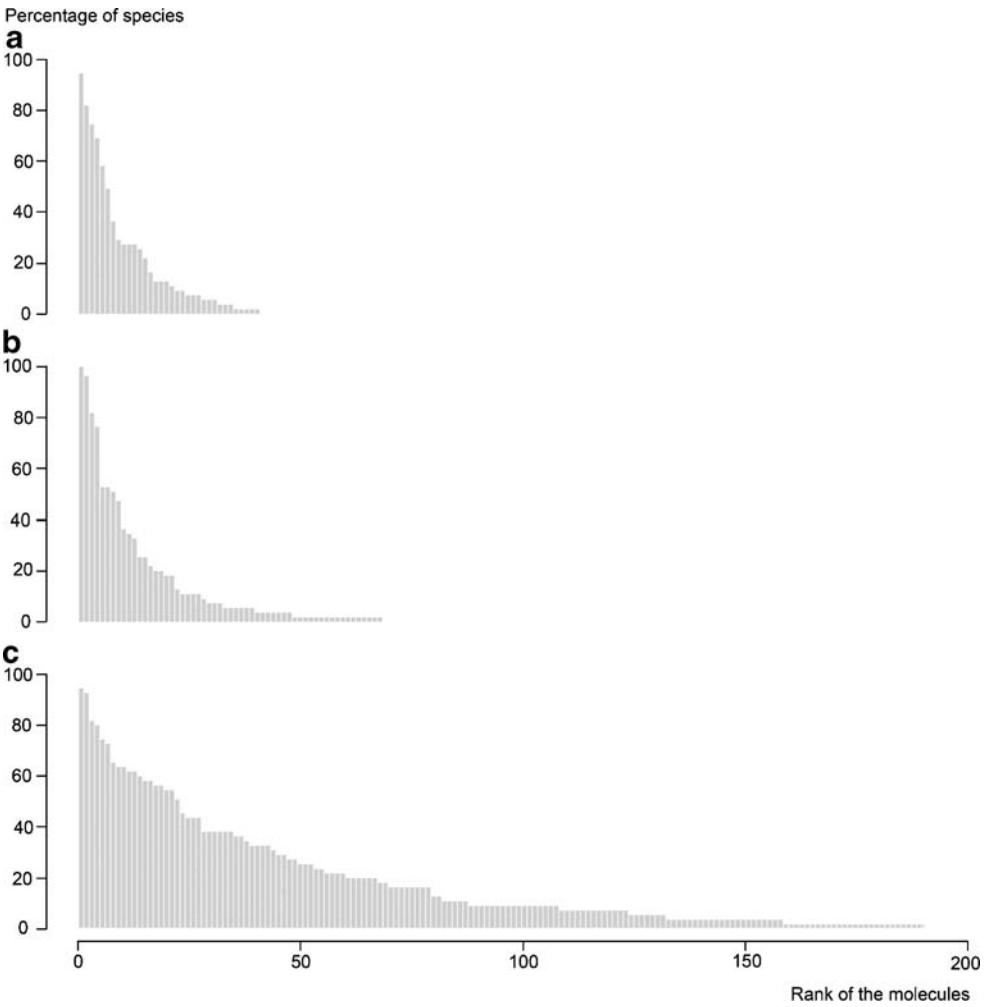

Fig. 2 Distribution of the VOCs isolated in the dataset with the percentage of species emitting each molecule for the three most common groups: **a** green leaf volatiles, **b** monoterpenes, and **c** sesquiterpenes

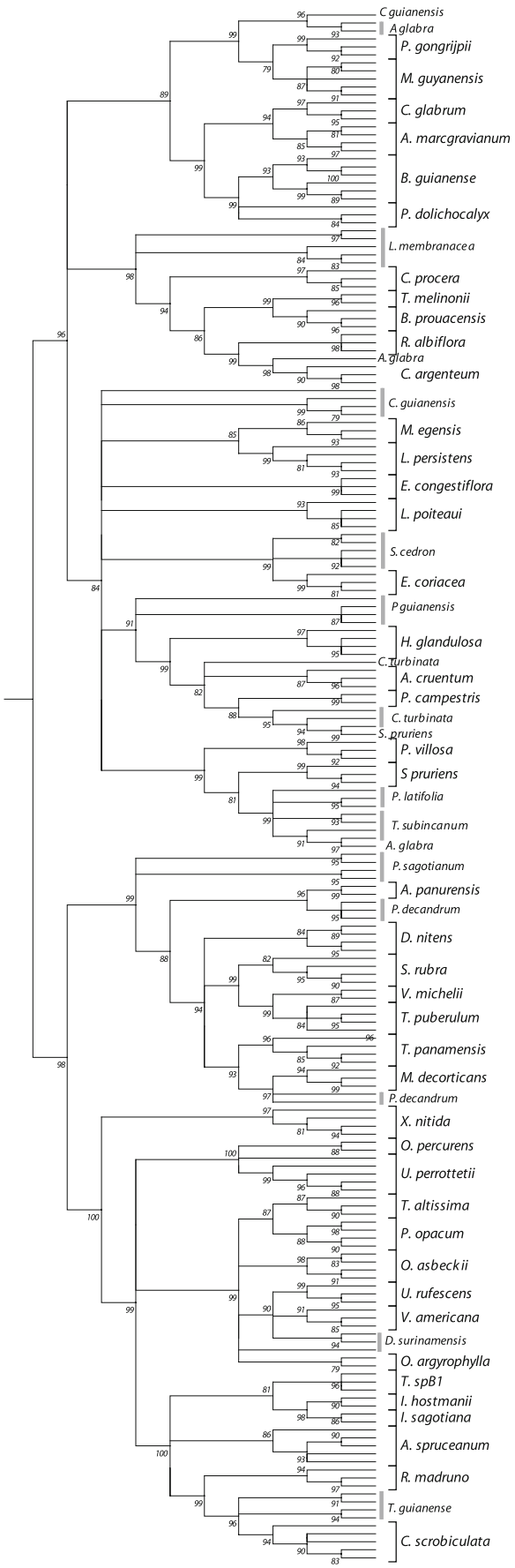

Fig. 3 Dendrogram displaying the dissimilarities in VOC composition among individuals, based on Ward clustering and Manhattan distance. Bootstrap support values greater than 80% on the nodes are reported on the tree. Species with names indicated in *grey* did not group into single clusters with a bootstrap value greater than 80%

(Kruskal-Wallis test, $P<0.001$). The mean number of VOCs per individual was 36.8 (Fig. 4). Chemical diversity was higher for species belonging to the Laurales, Sapindales, or Magnoliales (Fig. 4a), mean number of compounds per species in each order 43.8, 48.7, and 58.5, respectively). Two exceptions to this general trend were *Simaba cedron,* a member of the Simaroubaceae, and *Sextonia rubra*, in the Lauraceae. Both species had lower chemical diversity relative to other members of their families (Fig. 4a), mean number of compounds 17.4 and 33.5, respectively). Species belonging to the Ericales, Gentianales, and Malvales emitted less diverse mixtures of VOCs (21.3, 22.6, and 24.6, respectively). These differences were mainly due to differences in the number of sesquiterpenes (Fig. 4b).

Discussion

The most prevalent compounds that were identified in this study have defensive roles in model plant species, and they are among the most common compounds identified in floral scents (Knudsen et al. 2006). Within the monoterpenes, both α-pinene and limonene are toxic to fungi, bacteria, and insects (Miresmailli et al. 2006; Bakkali et al. 2008). These two compounds are known to be emitted in large quantities by tropical trees, as atmospheric chemists have previously detected them in high concentration in the atmosphere above Amazonian forests (Greenberg et al. 2004). Here, we demonstrated that a large array of species emit them. The other monoterpenes ((E)-β-ocimene, β-myrcene, linalool, β-pinene) and the irregular terpene (6-methyl-5-hepten-2-one), common in floral scents, are also found in vegetative emissions (Appendix 1). The sesquiterpenes caryophyllene and α-copaene have been implicated in direct and indirect responses against herbivores and pathogens (Heil 2004; Gols et al. 2008; Köllner et al. 2008), and they were distributed widely in the studied tree species.

The blend of VOCs is largely species-specific, as most species formed single clusters based on their VOC profiles. Taxa above the species level (genus, family, or order) usually were not characterized by a distinct profile. This result is in agreement with the pattern observed in floral scents where VOCs are consistent within a species but usually differ among closely related ones (Knudsen et al. 2006).

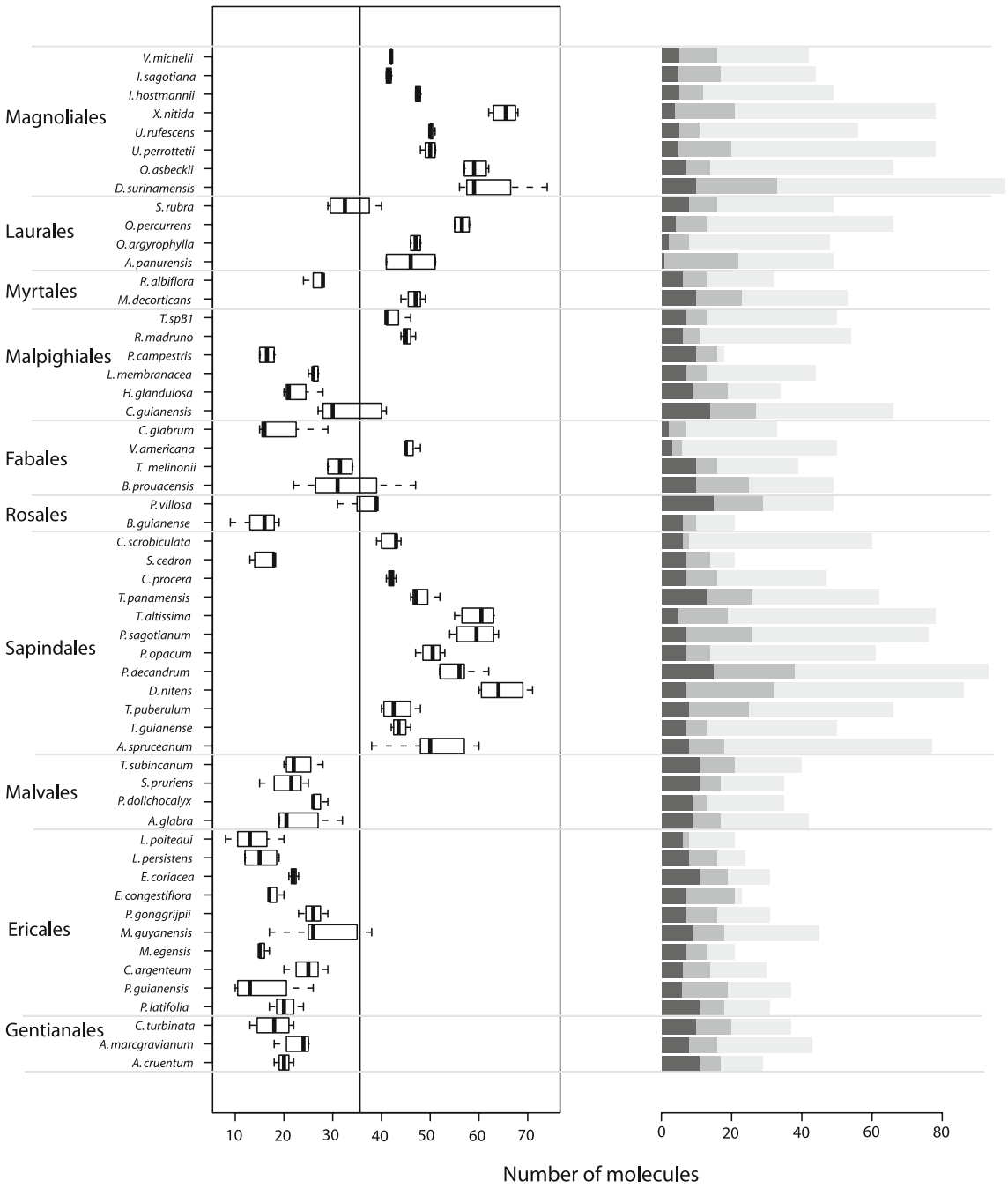

Fig. 4 **a** Mean number of compounds and the variation observed among conspecific individuals. For each box, *leftmost point* represents the 5% quantile and the *rightmost point*, the 95% quantile. The *rectangular box* represents the 25% quantile to 75% quantile ranges. The *dark line* shows the median of the distribution. The *vertical line* represents the mean number of compounds in the dataset (36.8) **b** contribution of the three more common class of compounds to the mean number of compounds for each species with green leaf volatiles in black, monoterpenes in *dark grey* and sesquiterpenes in *light grey*. *Horizontal lines* separate the distinct order in the dataset

The total diversity of VOCs varied greatly across species. Species in the Sapindales, Magnoliales, and Laurales generally released a more diverse blend than species in other orders. Exceptions were *Simaba cedron* (Simaroubaceae, Sapindales), and *Sextonia rubra* (Lauraceae, Laurales), which emit simpler mixtures of VOCs than other species in their respective families. *Simaba cedron* is actively defended by quassinoids, oxygenated triterpenes that are not detected by GC-MS (Ozeki et al. 1998). A non-volatile defensive compound has been isolated in great quantity from *S. rubra* wood (D. Stien, unpublished results). These specific examples suggest that plants

defended by other compounds may have simpler VOC profiles than plants that rely mainly on VOC defense.

Terpenoids made up the majority of the observed chemical diversity, consistent with our current knowledge on plant VOC synthesis (Wink 2003). Sesquiterpenes were more diverse than monoterpenes in tropical trees, with many compounds produced by only a few species. Diverse sesquiterpene composition is not the rule for all tree species: conifers emit mostly monoterpenes and only a few sesquiterpenes (Keeling and Bohlmann 2006). Our data suggest that production of sesquiterpenes may be more important than previously thought for tropical plants. In previous studies, VOC emissions from tropical forests have been quantified mostly in atmospheric studies of air pollutants (Guenther 2002). Generally, sesquiterpenes are difficult to detect in the air, due to their short lifetime in the atmosphere (Kesselmeier and Staudt 1999; Guenther 2002). Understanding the importance of sesquiterpene emissions by tropical forests remains an important challenge (Bouvier-Brown et al. 2007), and the present work provides a reference database for future investigations in this direction.

We hypothesize that plant defense by VOCs depends not only on the abundance of the various emitted VOCs, but also on the diversity of the blend. Fine et al. (2006) recently compared the investment in the production of monoterpenes and sesquiterpenes in tropical tree species to test the hypothesis that species under a higher herbivory pressure are better defended chemically. This hypothesis was not supported for the genus *Oxandra* (Annonaceae, Magnoliales). Our analysis may provides an explanation for their result: Fine et al. (2006) only compared overall concentration of terpenes and did not look at the diversity of the blend.

An assumption of our study is that, to measure the chemical diversity of VOC blends, we considered each compound as an independent unit. However, VOCs are linked by their biosynthetic pathways, especially in the sesquiterpene and monoterpene groups where terpene synthases often are involved in the synthesis of multiple products. An important challenge is to determine whether biosynthetic pathways may be partially elucidated by data from large-scale surveys.

One important limitation of our analysis is that we characterized the blend of VOCs released immediately after inflicting mechanical damage and not after an herbivory event. We assumed that this blend is representative of the constitutive VOCs released immediately after herbivore attack (see also Banchio et al. 2005; Mithöfer et al. 2005). However, VOC emissions are often modulated by factors such as elicitors released by herbivores (Mattiacci et al. 1995). In some species, VOCs are stored in specialized cells and volatilized immediately after wounding, whereas in others, synthesis is triggered by herbivory (Turlings and Wäckers 2004). Our study was designed to detect general pattern of constitutive VOCs among numerous long-lived species. Detailed studies will be necessary to understand fully the relative role of constitutive and induced VOCs in the defensive strategies of tropical trees.

In summary, our analysis highlights three major aspects of VOC emissions by tropical tree species. First, for most species, the intraspecific variation of the VOC blend is low, suggesting that VOC composition is species-specific. Second, sesquiterpenes are a key constituent of the VOC blend. Future studies should link pathogens and herbivore occurrence as well as herbivory rates with the quantity and diversity of sesquiterpenes in leaves and bark. Third, the complexity of the VOC mixtures varies significantly among species, indicating large differences in VOCs production among species. Future studies will need to relate this observation to our understanding of the evolution of plant defenses and to the herbivores and pathogens community associated with each species.

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Appendix 1

SPME fiber exposure time for leaves and bark of each species

species code	Bark extraction (min)	Leaf extraction (min)
D. surinamensis	15	15
O. asbeckii	15	15
U. perrottetii	15	15
U. rufescens	15	15
X. nitida	15	15
I. hostmannii	30	30
I. sagotiana	30	30
V. michelii	30	30
A. panurensis	5	15
O. argyrophylla	5	15
O. percurrens	5	15
S. rubra	5	15
M. decorticans	30	15
R. albiflora	30	30
C. glabrum	30	30
H. glandulosa	30	30

L. membranacea	60	30
P. campestris	60	30
R. madruno	30	30
T. spB1	15	15
C. guianensis	30	30
T. melinonii	15	30
V. americana	5	5
B. prouacensis	30	30
P. villosa	30	30
B. guianense	30	30
A. spruceanum	15	30
T. guianense	15	15
T. puberulum	15	15
D. nitens	5	15
P. decandrum	5	15
P. opacum	5	15
P. sagotianum	5	15
T. altissima	5	15
T. panamensis	5	15
C. procera	30	30
C. scrobiculata	15	15
S. cedron	60	15
P. dolichocalyx	30	30
S. pruriens	30	30
T. subincanum	30	30
A. glabra	30	30
E. congestiflora	30	15
E. coriacea	30	15
L. persistens	30	15
L. poiteaui	30	30
C. argenteum	30	30
M. egensis	30	30
M. guyanensis	30	30
P. gonggrijpii	30	30
P. guianensis	30	15
A. cruentum	30	30
A. marcgravianum	30	30
C. turbinata	30	30
P. latifolia	30	30

Appendix 2

Compounds tentatively identified. For each compound, the Kovats RI and the number of species that emits the compound is indicated. The compounds authenticated with standards are indicated by *.

classe	compound	RI	# species
N_compound	2-isopropyl-3-methoxypyrazine	1091	1
shikimic pathway	unknown S 1	1206	15
	methyl salicylate	1206	5
	1,4-dimethoxybenzene	1249	1
lipidic pathway (LP)	hexanal	813	52
	n-hexanol	872	45
	(*E*)-2-hexenal	863	41
	penten-3-ol	741	38
	2-ethyl furan	749	32
	(*Z*)-3-hexenol	864	27
	unknown LP 1	733	20
	isopentyl alcohol	768	16
	unknown LP 2	735	15
	octen-3-ol	984	15
	3-octanone	989	15
	unknown LP 3	854	14
	2-pentanone	743	12
	penten-1-ol	791	9
	3-pentanone	749	7
	heptanal	905	7
	hexyl hexanoate	1386	7
	(*E*)-3-hexenol	857	6
	1-pentanol	769	5
	hexenyl acetate	1014	5
	3-methyl-3-buten-1-ol	757	4
	3-octanol	1000	4
	hexenyl-3-methylbutanoate	1231	4
	(*Z*)-2-hexenol	872	3
	octanone	989	3
	hexenyl butanoate	1186	3
	octene	800	2
	(E)-2-octen-1-al	1066	2
	hexyl butanoate	1192	2
	unknown LP 4	805	1
	unknown LP 5	909	1
	unknown LP 6	970	1
	hexenyl isobutanoate	1142	1
	hexenyl isovalerate	1236	1
irregular terpene	6-methyl-5-hepten-2-one	989	1
monoterpene	α-pinene *	940	55
	limonene *	1034	53
	p-cymene	1030	45
	β-pinene	985	42
	3-carene	1014	29
	β-myrcene *	992	29
	α-phellandrene	1011	28
	camphene	958	26
	linalool *	1103	20
	p-xylene	881	19
	α-thujene	932	18
	β-phellandrene	1036	14
	β-terpinolene	1089	14
	1,8-cineole	1038	12

	α-terpinene	1022	11
	γ-terpinene	1062	11
	β-ocimene	1049	10
	sabinene	978	10
	o-xylene	905	7
	iso-methoxythymol	1241	6
	o-cymene	1025	6
	Perilene	1114	6
	tricyclene	930	6
	delta-2-carene	1002	5
	α-terpinolene	1083	4
	cis-sabinene-hydrate	1075	4
	terpinene-4-ol	1188	4
	allo-ocimene	1119	3
	linalool-oxide-trans	1075	3
	unknown monoterpene 1	1173	3
	mentha-1-7(8)-diene	1010	3
	p-cymenene	1096	3
	verbenene	986	3
	3p-menthene	1000	2
	camphor	1156	2
	carvacrol-methyl-ether	1232	2
	(*E*)-β-ocimene	1047	2
	mentha-2,8-diene	1001	2
	thuja-2,4(10)-diene	961	2
	thymol-methyl-ether	1227	2
	(4*E*,6*Z*)-allo-ocimene	1131	1
	α-terpineol	1202	1
	campholenal	1133	1
	cymen-8-ol	1194	1
	linalool-oxide-cis	1180	1
	linalool-oxide-dihydroxy	1112	1
	unknown monoterpene 2	1047	1
	mentha-2,8-dienol	1123	1
	myrtenal	1204	1
	pinocarvone	1170	1
	rose-furan-oxide	1197	1
	trans-sabinene-hydrate	1108	1
	sylvestrene	1021	1
	trans-pinocarveol	1149	1
	trans-verbenol	1152	1
	(*Z*)-β-ocimene	1035	1
sesquiterpene	β-caryophyllene *	1427	52
	α-copaene	1381	51
	α-humulene	1462	45
	δ-cadinene	1521	44
	germacrene D	1487	41
	cyperene	1412	40
	bicyclogermacrene	1502	36
	allo-aromadendrene	1467	35
	sesquithujene	1393	35
	α-ylangene	1375	34
	di-exo-T-cadinol	1479	34
	γ-cadinene	1518	33
	calarene	1436	32
	unknown sesquiterpene 1	1393	32
	aromadendrene	1445	31
	unknown sesquiterpene 2	1503	31
	α-cubebene	1349	30
	unknown sesquiterpene 3	1502	30
	trans-calamenene	1526	28
	δ-elemene	1338	25
	β-humulene *	1433	24
	bicyclo-elemene	1335	24
	eremophyladiene	1541	24
	α-selinene	1496	21
	caryophyllene-oxide	1592	21
	cis-cadina-1,4-diene	1498	21
	γ-elemene	1432	21
	unknown sesquiterpene 4	1350	21
	unknown sesquiterpene 5	1439	21
	α-cadinene	1536	20
	β-bazzanene	1529	20
	(*Z*)-α-bisabolene	1509	19
	α-muurolene	1507	18
	unknown sesquiterpene 6	1399	18
	unknown sesquiterpene 7	1435	18
	sesquithujene-7-epi	1389	18
	gamma-selinene	1479	17
	allo-aromadendra-4(15),10(14)-diene	1455	16
	sesquiphellandrene	1514	16
	african-2(6)-ene	1361	15
	α-cedrene	1416	15
	7-epi-α-cedrene	1404	14
	α-guaiene	1439	14
	unknown sesquiterpene 8	1450	14
	1-epi-α-pinguisene	1370	13
	β-maaliene	1417	13
	(*Z*)-β-farnesene	1451	12
	β-bisabolene	1503	12
	unknown sesquiterpene 9	1483	12
	viridiflorene	1496	12
	anastreptene	1370	11
	g-muurolene	1480	11
	iso-carryophyllene	1411	11
	rotundene	1469	11
	unknown sesquiterpene 10	1327	11
	unknown sesquiterpene 11	1434	11
	β-calacorene	1547	10
	β-curcumene	1512	10
	α-curcumene	1484	9
	δ-selinene	1492	9
	unknown sesquiterpene 12	1500	9

unknown sesquiterpene 13	1528	9
unknown sesquiterpene 14	1547	9
unknown sesquiterpene 15	1369	9
unknown sesquiterpene 16	1405	9
unknown sesquiterpene 17	1451	9
β-cubebene	1385	7
β-selinene	1490	7
(*Z,E*)-α-farnesene	1488	6
β-elemene	1385	6
unknown sesquiterpene 18	1325	6
spathulenol	1585	6
trans-cadina-1,4-diene	1538	6
7-epi-α-selinene	1534	5
α-longipinene	1358	5
β-barbatene	1458	5
β-bourbonene	1390	5
β-ylangene	1424	5
cadina-3,5-diene	1454	5
calameren-9-ol	1555	5
cis-calamenene	1532	5
cuparene	1517	5
gorgonene	1446	5
hinesene	1528	5
muurolol	1603	5
oppositadiene	1393	5
presilphiperfolene	1312	5
unknown sesquiterpene 19	1507	5
unknown sesquiterpene 20	1443	5
unknown sesquiterpene 21	1425	5
α-gurjunene	1413	4
β-acoradiene	1472	4
bourboneral	1555	4
cadinene-ether	1570	4
γ-curcumene	1480	4
guaiadiene	1407	4
maali-1,3-diene	1351	4
unknown sesquiterpene 22	1530	4
unknown sesquiterpene 23	1420	4
unknown sesquiterpene 24	1449	4
selina-4,7-diene	1513	4
striatene	1461	4
trans-cubebol	1514	4
(*E*)-β-farnesene	1461	3
aromadendra-4,10(14)-diene	1442	3
cadina-1(10),6-diene	1461	3
epi-α-muurolol	1655	3
unknown sesquiterpene 25	1455	3
unknown sesquiterpene 26	1484	3
unknown sesquiterpene 27	1458	3
α-alaskene	1514	2
α-cadinol	1666	2
α-santalene	1423	2
bourbon-11-ene	1429	2
brasiladiene	1336	2
cubenol	1624	2
cyclo-bazzanene	1523	2
cyperadiene	1358	2
dendrolasine	1576	2
epi-α-cadinol	1654	2
gamma-guaiene	1511	2
isoledene	1378	2
unknown sesquiterpene 28	1457	2
unknown sesquiterpene 29	1523	2
unknown sesquiterpene 30	1437	2
unknown sesquiterpene 31	1328	2
unknown sesquiterpene 32	1389	2
unknown sesquiterpene 33	1509	2
unknown sesquiterpene 34	1533	2
unknown sesquiterpene 35	1443	2
unknown sesquiterpene 36	1444	2
selina-4,11-diene	1482	2
(3*E*,6*Z*)-α-farnesene	1481	1
5-epi-aristolochene	1476	1
african-2,6-diene	1345	1
α-cuprenene	1555	1
α-duprezzianene	1389	1
β-chamigrene	1534	1
β-vetivene	1536	1
brasila-1(6),5(10)-diene	1436	1
cadalene	1635	1
calamenol	1550	1
cymene-2,5-dimethoxy-para	1415	1
(*E*)-γ-bisabolene	1529	1
epistolene	1393	1
erythrodiene	1447	1
2-epi-α-funebrene	1419	1
germacrene B	1567	1
iso-bicyclogermacrene	1489	1
pacifigorgia-1(9),10-diene	1385	1
pacifigorgia-2,10-diene	1431	1
palustrol	1581	1
unknown sesquiterpene 37	1462	1
unknown sesquiterpene 38	1493	1
unknown sesquiterpene 39	1577	1
unknown sesquiterpene 40	1585	1
unknown sesquiterpene 41	1422	1
unknown sesquiterpene 42	1334	1
unknown sesquiterpene 43	1343	1
unknown sesquiterpene 44	1496	1
unknown sesquiterpene 45	1424	1
unknown sesquiterpene 46	1448	1
unknown sesquiterpene 47	1329	1
unknown sesquiterpene 48	1506	1
unknown sesquiterpene 49	1420	1

unknown sesquiterpene 50	1423	1
sesquicineole	1516	1
veltonal	1595	1
widrene	1441	1

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levels in *Nicotiana sylvestris* Spegazzini and Comes through wounding and removal of leaves varying in age (young, mature, and old) and found that changes in nicotine distribution and concentration were consistent with ODT.

Iridoid glycosides are secondary metabolites that have been isolated and identified from a large number of plant species, but relatively little is known of their distribution and function within plants. A body of previous work focused on the defensive function of iridoids from narrow-leaved plantain (*Plantago lanceolata* L.), which has two major iridoids, catalpol, and aucubin (Bowers and Puttick 1986, 1988; Puttick and Bowers 1988). More recent work on *P. lanceolata* has shown that catalpol is the predominant iridoid in young to intermediate aged leaves, with older leaves containing mainly aucubin (Bowers and Stamp 1992; Klockars et al. 1993; Fuchs and Bowers 2004). Catalpol was found to be a more potent growth inhibitor of larval *Spodoptera eridania* (Stoll) than aucubin (Puttick and Bowers 1988), and its high concentration in younger leaves is consistent with ODT. The structural similarity and defensive function of catalpol in *P. lanceolata* as compared to antirrhinoside of *Antirrhinum majus* L. has been described by Beninger et al. (2008).

Antirrhinum majus (snapdragon) is native to the Mediterranean region (Sutton 1988), and based on genetic analyses recently has been placed in the family Plantaginaceae along with *Linaria vulgaris* Mill. (yellow toadflax) (Albach et al. 2005). According to one of the most parsimonious trees of the combined analysis of common genes, *Antirrhinum* and *Linaria* are placed in the subfamily Antirrhineae (Albach et al. 2005). *Linaria vulgaris* is native to Eurasia but was introduced into North America over 300 yr ago. It escaped cultivation and has since become well established as a noxious weed (Saner et al. 1995). *Antirrhinum majus* reproduces solely through the production and dispersal of seed. *Linaria vulgaris* reproduces through seed but also asexually by means of radical shoots produced from buds that arise endogenously from the main, or long, horizontal roots (Bakshi and Coupland 1960; Charlton 1966; Sutton 1988). Vegetative propagation becomes an important mode of reproduction for *L. vulgaris* seasonally and at high latitudes (Nadeau and King 1991; Nadeau et al. 1992). For example, *L. vulgaris* plants that were introduced in grain shipments to a sub-arctic habitat (Churchill, Manitoba, Canada, 58° 46′ L.N), have become frequent and persistent, but they do not produce viable fruit, and they spread exclusively through radical shoots (Staniforth and Scott 1991).

Iridoid glucosides are common to both *A. majus* and *L. vulgaris* (Scarpati et al. 1968; Guiso and Scarpati 1969; Scarpati and Guiso 1969; Sticher 1971; Ilieva et al. 1992, 1993). However, for *A. majus*, we have found that antirrhinoside is the predominant iridoid in all plant tissues except for older leaves that contain mainly antirrhide (Beninger et al. 2007). Similarly, for *L. vulgaris*, antirrhinoside is the major iridoid found in all plant tissues (this paper). Antirrhinoside, is known to be phloem mobile in *Asarina scandens* (Cav.) Penn. (Gowan et al. 1995), and recent research has shown that this also is likely the case for antirrhinoside in *A. majus* (Beninger et al. 2007). Other possible defensive secondary metabolites such as the alkaloids choline (Harkiss 1974) and linarinic acid (Hua et al. 2002) have been found in *A. majus* and *L. vulgaris*, but their concentrations are extremely low (9.7×10^{-4} % and 2.5×10^{-6} %, respectively). Whether they have biological activity at these concentrations is unknown. However, antirrhinoside, can be found in concentrations ranging from 5 to 23% dry weight of plant tissue in *A. majus* (Beninger et al. 2007), and is known to inhibit growth of larval gypsy moth significantly at a concentration of only 3.3% in artificial diet (Beninger et al. 2008).

The importance of sexual reproduction to *A. majus* and of both sexual and asexual reproduction for *L. vulgaris* led to the generation of questions that could best be framed in the context of ODT. For example, constitutive levels of antirrhinoside are known to vary with time in organs of *A. majus*; young leaves and flowers contain the highest levels of antirrhinoside, and roots the lowest. How do these patterns of distribution compare to a species such as *L. vulgaris*, which also has antirrhinoside as its predominant iridoid, but for which sexual or asexual reproduction may differ in importance throughout ontogeny? Specifically, are new leaves of *L. vulgaris* protected with levels of antirrhinoside comparable to *A. majus*, and do these levels change in a similar manner during ontogeny? Since roots are a reproductive organ for *L. vulgaris*, how do root levels of antirrhinoside compare to *A. majus*, and do these levels change with time? How does allocation of antirrhinoside to flowers, the only reproductive organs for *A. majus*, compare to *L. vulgaris* which has both flowers and radical shoots as reproductive organs?

Methods and Materials

Seed Reproduction of A. majus and L. vulgaris The average seed production per capsule of the *A. majus* cv. Maryland White Ivory used in this study is 47 with ca. 73–85% germination rate (*pers. comm.* Linda Laughner, plant breeder, PanAmerican Seed™, Chicago, IL, USA). For *L. vulgaris*, seed production is poor due to pollinator limitation (Arnold 1982), predation (McClay 1992), and resource limitation (Clements and Cavers 1990). Average numbers of seed per capsule in field collections varied between 24.5 and 38.6 (Clements and Cavers 1990) with seed germination of only 8±1% to 53±19% (Nadeau and

King 1991). Seed dispersal also is poor (Nadeau and King 1991), and seed can have strong dormancy (Andersen 1968).

Plant Material and Environmental Conditions Seeds of *A. majus* Maryland Ivory White cultivar provided by Pan-American Seed™ (Chicago, IL, USA) were sown in 128 cavity plug trays (Landmark Co. Plastic, Akron, OH, USA) Sept. 21, 2005 in soil-less growth medium (Pro-Mix®, Premier Horticulture Ltée/LTD Rivière du Loup, QC, Canada) and grown until Nov. 04 2005, initially in a glass greenhouse (University of Guelph, ON, Canada, 43.3° 15′ L.N.) under ambient light at a temperature of 22/15 °C (day/night). *Linaria vulgaris* seeds purchased from Chiltern Seeds (Cat.No.813E; Cumbria, UK) were sown Nov. 17, 2006 and grown until Jan. 09, 2007 under the same temperature and greenhouse conditions described for *A. majus*, except that when selecting seedlings for transplant into hydroponic solution, those with one or two initial stems were chosen preferentially over those with more stems. The second stem (ramet), if present, was smaller, and was pinched off to ensure uniformity before the plants were grown hydroponically in the growth chambers. The removal of the small ramet is similar to defoliation of one of a pair of ramets, and when this was done for *L. vulgaris*, there was no difference in the total amount of ^{13}C labeled in the two ramets (Hellström et al. 2006). It is, therefore, unlikely that pinching of the small ramet had any effect on antirrhinoside concentration in the remaining stem.

Both species were removed from the growth medium, roots washed with distilled water, and then grown hydroponically in environmental chambers as described in Beninger et al. (2007). Briefly, seedlings of both species were transplanted to 1.8 liter containers filled with aerated nutrient solution as described by Johnstone et al. (2005). To exclude light from the nutrient solution, which could promote the growth of algae, the containers were inserted into a larger black plastic container, and its lid was covered with black-on-white plastic sheeting, white side upwards. Hydroponic single-plant units were positioned in a growth chamber with a 12-h photoperiod and provided 350±50 μmol $m^{-2}s^{-1}$of photosynthetically active radiation (PAR) at plant canopy height. Day/night temperatures were approximately 23.5/18°C. Plants were fertilized with a commercial hydroponic formulation (1.15 g of 6-11-35 N:P:K supplemented with 0.85 g of $CaNO_3$ per liter, Plant Products Ltd., Brampton, ON, CA), with a pH of 6.0, and electrical conductivity (EC) of 1.9 mS/cm. Each main stem was supported by a galvanized wire stake (gauge 12). Seedlings received a half strength nutrient solution for 5 d after transplant. Thereafter, seedlings received full strength nutrient solution, and the solution level was maintained during the experiment. *Antirrhinum majus* cultivars have been grown hydroponically in our laboratory to commercial flower maturity previously (Ortiz-Uribe et al. 2005), but this is the first time that *L. vulgaris* has been grown successfully in hydroponics.

For the sampling of leaf tissue, there are two types of leaves in *A. majus* and *L. vulgaris*, axillary and lateral. Axillary leaves are first to develop and are attached directly to the main stem. Lateral leaves are attached to lateral stems that branch from the main stem above the axillary leaves (Figs. 1 and 2). Axillary leaves and lateral stems originate from slightly enlarged areas of the main stem, defined as nodes. Axillary leaves were harvested from the 4th, 8th, and 12–14th node from the base. Lateral leaves harvested were the 1st pair of leaves on the lateral stem at the 4th and 8th node of the main stem (see Fig. 3 in Beninger et al. 2007 for a diagram). Sampling the leaves at different nodes was done to obtain leaves from youngest (12th–14th node) to oldest (4th node) parts of the plants.

Plant organs for *A. majus* were harvested between 1300–1430 h 44, 54, 61, 68, 75, 82, and 89 days after sowing (DAS). Harvests for *L. vulgaris* occurred between 1330–1500 h at the following DAS: 53, 61, 68, 75, 82, 89, and 94. Harvesting of *A. majus* was ended at 89 DAS because the flower spike was fully developed and plants had reached commercial maturity. Harvesting of *L. vulgaris* was terminated at 94 DAS because some of the flowers at the lower portions of the inflorescence had begun to senesce. Three replicate plants per species were harvested at each of the above time periods. Leaves were removed at the stem, wrapped in aluminum foil, immediately frozen in liquid N_2, stored in a −80°C freezer, and freeze-dried. Non-leaf tissue was separated according to organ type (i.e. stem, root, etc.) and then treated as the leaves. Prior to HPLC analysis,

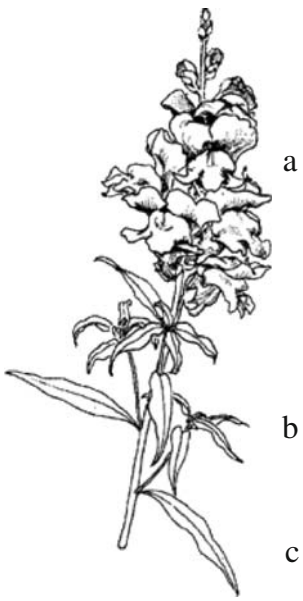

Fig. 1 Growth habit and flower morphology of *Antirrhinum majus.* **a.** Flower **b.** Lateral Leaves **c.** Young axillary leaf. Drawing modified from University of Florida Fact Sheet FPS-44

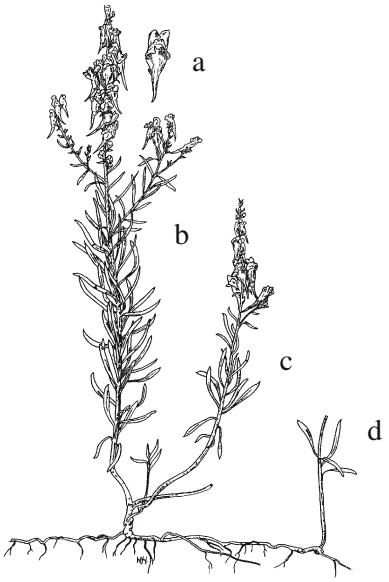

Fig. 2 Growth habit and flower morphology of *Linaria vulgaris*. **a.** Flower **b.** Lateral Leaves **c.** Clonal Growth from Main Root **d.** Clonal Growth from Lateral Root. Drawing modified from Saner et al. (1995)

freeze dried tissue of *L. vulgaris* was prepared and extracted as described for *A. majus* in Beninger et al. (2007).

HPLC Instrument and Conditions Instruments and conditions for analysis of *A. majus* extracts were as described in Beninger et al. (2007). Briefly, an Agilent model 1100 HPLC equipped with a degasser, quad pump, autosampler, and diode array display (DAD) (Agilent Technologies Canada Inc., Mississauga, ON, CA) was used. The column used was a CapCell Pak AG 120 C18 (4.6×250 mm, 5μ particle size) (Shiseido Co. Ltd. Japan), and the HPLC had ChemStation software. For quantification of the iridoids, the solvent system was isocratic employing 6% acetonitrile in water with a flow rate of 1.0 ml/min and sample elution periods of 10 min for *A. majus*. However, for *L. vulgaris*, the solvent composition was changed to 8% acetonitrile in water to decrease run times, since there was no closely eluting antirrhide to interfere with the separation and quantification of antirrhinoside. Water and acetonitrile were both HPLC grade.

Statistical Analyses All data were analyzed using the SAS® (2005) statistical software package. The data for antirrhinoside concentrations for both species was square-root transformed, and the total data set passed normality tests. Data were analyzed by using regression analysis in Figs. 3 and 4 with r^2 and P values given for the regression lines on the graphs. However, when divided into species, organ, and harvest, and analyzed separately for normality, there were three cases in which the data were not distributed normally, and univariate ANOVA analysis would not be appropriate. Therefore, in order to determine significant differences in antirrhinoside concentration between the two species for individual plant organs at each harvest, non-parametric Wilcoxon rank-sum tests were used. Significant differences at $P<0.05$ are indicated by stars above the points at different harvests on the graphs in Figs. 3 and 4. The data presented in Table 1 passed normality tests and was analyzed by using 1-way ANOVAs. Although the data were analyzed using the square-root transformed values, actual means and standard errors are depicted in the figures and table.

Results

Comparison of Antirrhinoside Levels in Organs of *A. majus* and *L. vulgaris*

Axillary Leaves. For node 4 (oldest leaves), the concentration of antirrhinoside at harvest 0 (at time of transplant to hydroponics) was high and not significantly different between the two species (Fig. 3a). However, by the second harvest and until the final harvest, the concentrations of antirrhinoside in *L. vulgaris* for leaves at this node were significantly higher (Fig. 3a). The regression analysis showed that the decline in antirrhinoside was only marginally significant for *L. vulgaris* but highly significant for *A. majus* (Fig. 3a). For node 8 (Fig. 3b), the only significant difference at a given harvest between the two species was at harvest 3 where *A. majus* had significantly more antirrhinoside than *L. vulgaris*. Regression analysis showed no significant decline in antirrhinoside for *L. vulgaris* but a significant decline for *A. majus* (Fig. 3b). At node 12-14, significantly higher amounts of antirrhinoside were found for *A. majus* at all harvests (Fig. 3c). Similar to leaves at node 8, regression analysis for leaves at node 12-14 showed no significant reduction in antirrhinoside for *L. vulgaris* but the decline for *A. majus* was significant (Fig. 3c).

Stems, Lateral Stems, Roots and Flowers The pattern of accumulation of antirrhinoside in the main stems is similar between the two species, but at harvests 4, 5, and 6, *A. majus* had significantly more antirrhinoside (Fig. 4a). The lateral stems of both species began with relatively high concentrations of antirrhinoside at the second harvest, and then both gradually declined with time (Fig. 4b). There was no significant difference in antirrhinoside concentration in the lateral stems between the two species at a given harvest, but the regression analysis showed that the decline for both was significant. The antirrhinoside concentration in the roots of *L. vulgaris* was significantly higher than *A. majus*

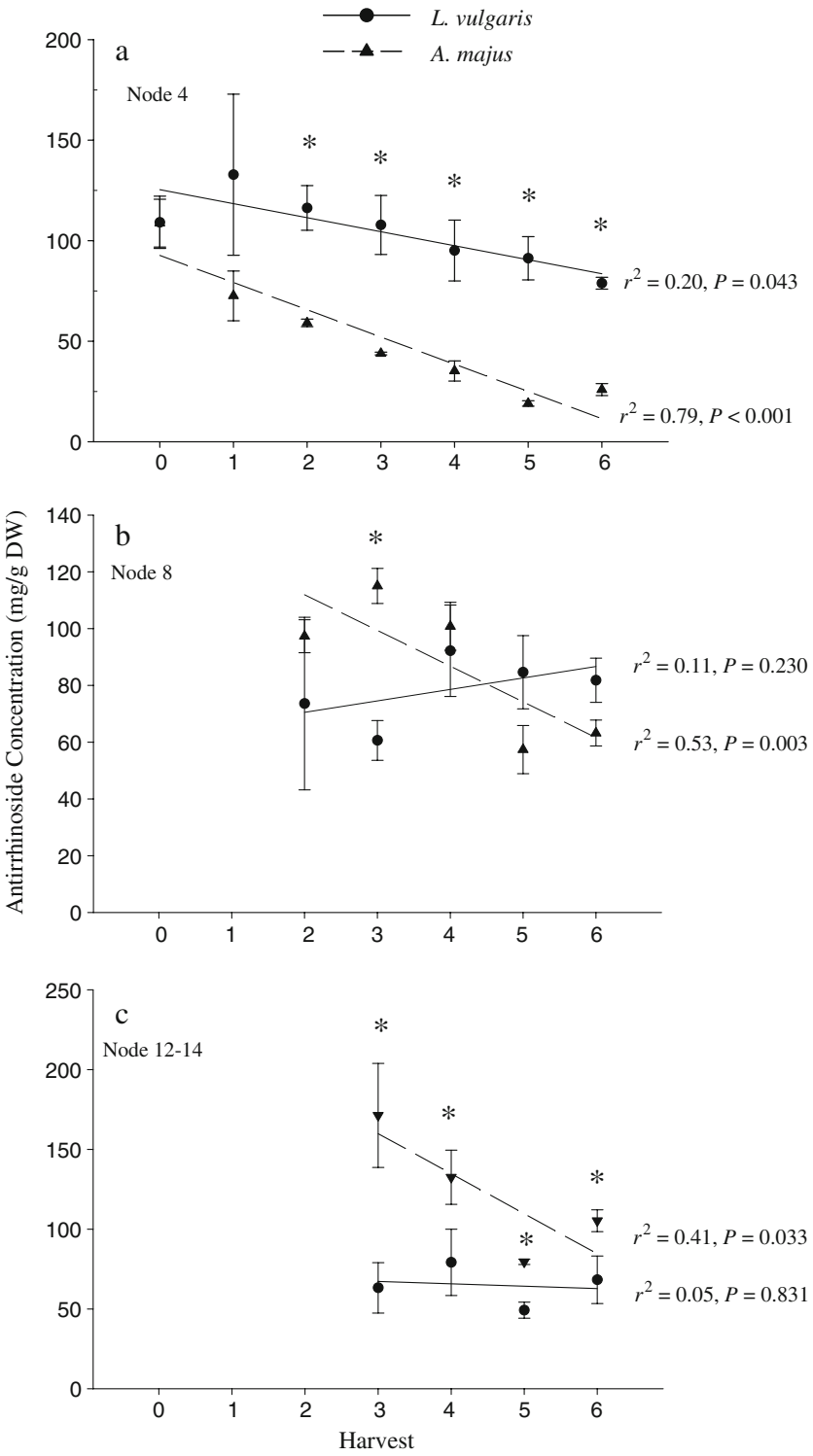

Fig. 3 **a.** Comparison of antirrhinoside concentrations for leaves of *Antirrhinum majus* and *Linaria vulgaris* at node 4 (oldest leaves) over the experimental period. Stars indicate significant differences between the two species ($P<0.05$). **b.** Same as for **a**, except means are for leaves at node 8 (intermediate aged leaves). **c.** Same as for **a** and **b**, except means are for leaves at node 12-14

at all harvests except for the initial one when the plants were removed from the growth medium (Fig. 4c) (Note: initial data points open circle and open triangle were not used in the regression analysis since these roots were analyzed immediately after removal from the soil-less growth medium, and may not give an accurate indication of antirrhinoside amounts in roots for that medium compared to roots grown in the hydroponic system). *Linaria vulgaris* still had over twice as much antirrhinoside as *A. majus* in this tissue by harvest 6. Antirrhinoside concentrations were very high in the buds of both species, but there was no significant difference between them. During the flowering

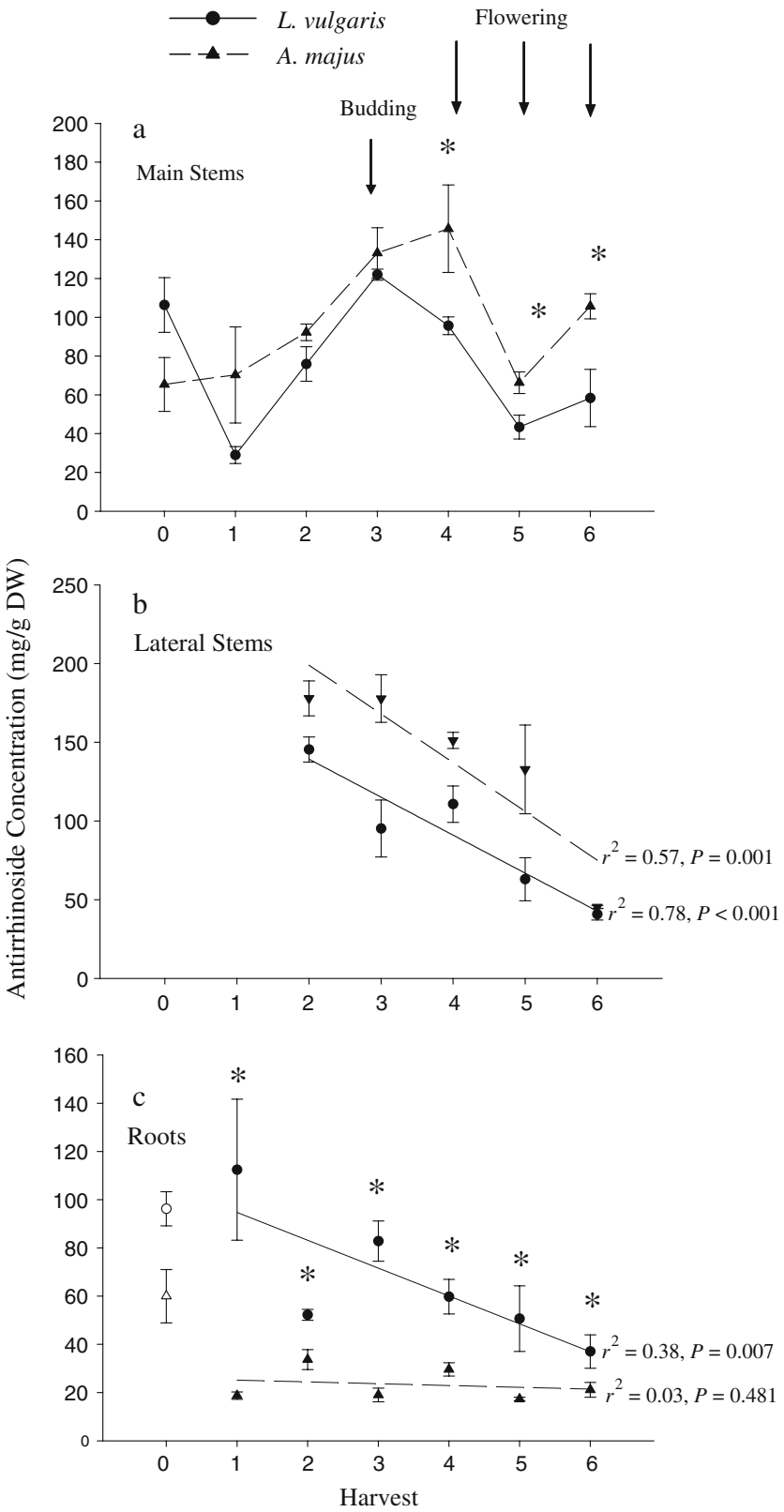

Fig. 4 **a.** Comparison of antirrhinoside concentrations in main stems of *Antirrhinum majus* and *Linaria vulgaris* over the experimental period. Stars indicate significant differences observed between the two species ($P < 0.05$). **b.** Same as for **a** except that means are for the lateral stems. **c.** Comparison of antirrhinoside concentrations for roots of *A. majus* and *L. vulgaris* over the experimental period

Table 1 Comparison between species in the levels of antirrhinoside (mg/g DW), in different organs of *Antirrhinum majus* and *Linaria vulgaris* over the flowering period

	Buds	Roots	Flowers
A. majus	195.42 (26.90)	22.71 (2.18)	142.10 (5.20)
L. vulgaris	169.69 (9.09)	49.17 (4.04)	96.93 (10.63)
P – value	0.5127	<0.001	<0.01

Actual means with SE in brackets are shown whereas the square root-transformed means were compared and significant differences between species indicated at $P<0.05$.

stage, *A. majus* had significantly higher concentrations of antirrhinoside in the flowers than *L. vulgaris* (Table 1).

Comparison of Biomass Allocation During the vegetative stage, *L. vulgaris* allocated significantly more relative biomass to axillary leaves than *A. majus*, but there was no difference between the species at the flowering stage (Fig. 5a). There was no difference between the two species in the amount of relative biomass allocated to lateral leaves at the vegetative stage, but at the flowering stage *L. vulgaris* allocated a significantly larger percentage to those leaves (Fig. 5b). During vegetative and flowering stages, *A. majus* allocated significantly more relative biomass to the main stem than *L. vulgaris*. In contrast, *L. vulgaris* allocated more to lateral stems at both stages than *A. majus*. There was an almost equal allocation of biomass for both species to root tissues at the two stages of development. Finally, *A. majus* allocated over two times the proportional amount of biomass to flowers as *L. vulgaris*.

Discussion

Antirrhinoside is structurally similar to catalpol found in *Plantago lanceolata*, which has been shown to reduce the growth of the generalist insect *Spodoptera eridania* (Puttick and Bowers 1988). We have shown that antirrhinoside reduces the growth of another generalist, gypsy moth, at concentrations in artificial diet well below those found in leaves (Beninger et al. 2008). Antirrhinoside, therefore, can be classified as a defensive compound, and predictions can be made about its distribution and concentration within plants according to ODT. Young, expanding leaves are most likely to be attacked by herbivores because of their higher nutritional value (Coley 1983), yet these are more valuable to the plant than older leaves due to their higher photosynthetic rates (Harper 1989), and according to ODT should contain higher levels of defensive compounds (Zangerl and Rutledge 1996). For *A. majus*, initial constitutive concentrations of antirrhinoside for all axillary leaves were high when young, and then tended to decline significantly with age (Fig. 3) consistent with ODT predictions. High concentrations of defensive compounds in young leaves also have been reported for terpenoid aldehydes in cotton (*Gossypium hirsutum* L.) (Anderson and Agrell 2005), pyrrolizidine alkaloids in *Cynoglossum officinale* L. (Van Dam et al. 1996), and phenolics in three tropical species (Read et al. 2003).

For *L. vulgaris*, a marginally significant decline in antirrhinoside over time was apparent only for leaves at node 4, and there was no significant reduction in this compound for the other axillary leaves (Fig. 3a–c). Even though the decline in antirrhinoside is significant for the oldest leaves of *L. vulgaris*, it is still significantly less than the decline in leaves of *A. majus* removed from the same

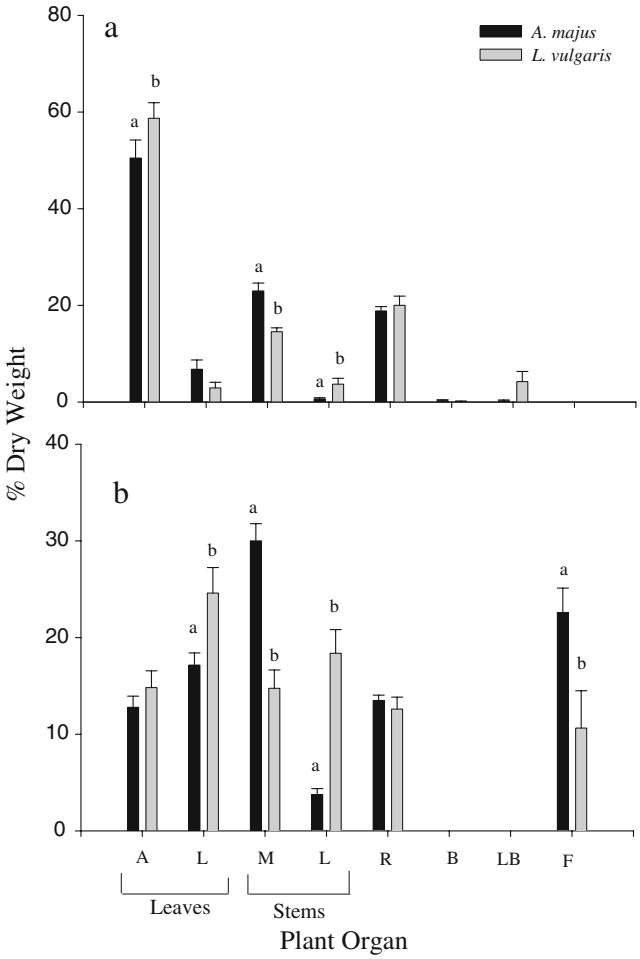

Fig. 5 a. Comparison between species of the % dry weight of different organs for *Antirrhinum majus* and *Linaria vulgaris* averaged over the vegetative period. Different letters above bars indicate significant differences between species at $P<0.05$. A = Axillary; L = Lateral; M = Main; R = Roots; B = Buds; LB = Lateral Buds; F = Flowers **b.** Same as for **a** except comparison is averaged over the flowering period

node. It is uncertain why the same pattern of defense allocation in young leaves of *L .vulgaris* generally is not seen, but species differences in leaf size and shape and plant architecture may affect the amount of light that is received by old vs. young leaves. It has been shown for a number of plant species that there is a decrease in net production with leaf age due both to senescence and to the over shading of older leaves by young leaves (Mooney and Gulman 1982; Harper 1989), which makes older leaves less valuable to the plant. Leaves of *L. vulgaris* are much smaller and narrower than leaves of *A. majus* and generally the same (homoblastic), regardless of where they are in the canopy. Axillary leaves of *A. majus*, are larger and heteroblastic; near the top of the plant, leaves are narrow but increase in width towards the bottom of the canopy. These differences in leaf morphologies and growth habits (architecture) of the two species (Figs. 1 and 2) would likely affect the amount of light older leaves receive, and this then would affect photosynthesis and the relative value of leaf tissue. In our study, however, we did not measure the amount of light received by leaves of different ages, and further research is needed to resolve this issue.

Accumulation of antirrhinoside in the main stem was remarkably similar between the two species with amounts peaking at bud-break for *L. vulgaris* or flowering for *A. majus* (Fig. 4a). Antirrhinoside has been shown to be phloem mobile when it occurs in other species (Gowan et al. 1995; Voitsekhovskaja et al. 2006), and we have shown through steady-state $^{14}CO_2$ labeling that it also is phloem mobile in *A. majus* as well (Beninger et al. 2007). There are no data on whether antirrhinoside is phloem mobile in *L. vulgaris*, but given the similar pattern of accumulation in the main stem it seems likely that it also is transported in the phloem. The ability to move antirrhinoside through the phloem has important implications in terms of its distribution as a defensive compound. For example, increases in leaf concentrations in antirrhinoside may not be solely a result of local induction, but rather reallocation from undamaged leaves or other parts of the plant. If the iridoid catalpol in *P. lanceolata* is also phloem mobile, this may be one of the reasons why local induction of iridoids is difficult to detect (Fuchs and Bowers 2004).

The lateral stems of both species (Fig. 4b) have a pattern of accumulation different from the main stems with initial high concentrations of antirrhinoside declining to less than half these values by the end of the experimental period. Antirrhinoside concentration in the lateral stems is high initially, perhaps because lateral leaf growth for both species declines with time. Since there were not enough lateral leaves of either species to analyze chemically over the experimental period, we do not know if the concentration of antirrhinoside in young lateral leaves of either species is high. However, lateral growth may be more important for *L. vulgaris* because it allocates more biomass on a relative basis to lateral stems and leaves during the flowering period (Fig. 5).

Root tissue is important for vegetative reproduction of *L. vulgaris* and if antirrhinoside is effective against root herbivores, then antirrhinoside concentrations in root tissue of *L. vulgaris* would be expected to have higher concentrations than *A. majus* root tissue. According to ODT the value of root tissue is higher for *L. vulgaris* than *A. majus* since its loss would have greater impact on the fitness of the former. However, for *L. vulgaris*, during flowering, the value of root tissue may decline and levels of root antirrhinoside also would be expected to decline as the flowers develop and become important for reproduction. This seems to be true for *L. vulgaris*, as root concentrations of antirrhinoside are initially about five times as high compared to *A. majus* and then steadily decline, whereas the concentration of antirrhinoside for *A. majus* remains low and constant throughout growth (Fig. 4c). It is noteworthy that the growth of these two plant species in a hydroponic medium is unique in that it allows for sampling of the root systems without the problems of accessibility and damage that results from removal of plants grown in a soil medium. On the other hand, hydroponic growth is artificial, and while clonal growth was observed for *L. vulgaris*, it may have been restricted by experimental conditions (pot size, shading) when compared to soil-grown plants.

There are, to our knowledge, no studies that have quantified root concentrations of antirrhinoside or other iridoids with the exception of a recent one by De Deyn et al. (2009). These authors examined iridoid distribution in *P. lanceolata* genotypes selected for high and low leaf concentrations of catalpol and aucubin, and their effects on root colonization by arbuscular mycorrhizal fungi (AMF). Concentrations in leaves and roots of the two iridoids were found to be positively correlated, and growth of AMF was negatively correlated with the concentration of root aucubin. These data do not support our previous suggestion that aucubin of *P. lanceolata* may be similar to antirrhide of *L. vulgaris*, i.e. it is not phloem mobile and found only in leaves (Beninger et al. 2007, 2008). De Deyn et al. (2009) reported the presence of aucubin in roots at concentrations of 0.05–0.93% DW depending on whether the plant was a high or low iridoid genotype. Further work on the distribution of catalpol and aucubin in *P. lanceolata* is needed to confirm these findings, but if aucubin is indeed in roots of *P. lanceolata*, it may function in defense.

According to ODT, flowers should be constitutively well defended, but not as inducible as other, less expendable tissues, such as older leaves. This has been shown to be the case for a toxic furanocoumarin in wild parsnip (*Pastinaca sativa*) (Zangerl and Rutledge, 1996) and to some extent for

glucosinolates in wild radish (Strauss et al. 2004, but see also Pavia et al. 2002). In our study, we did not test whether antirrhinoside was inducible in flowers or any other tissues, but we did measure the constitutive concentrations over plant ontogeny. During the flowering period of both species, allocation of antirrhinoside was significantly higher in flowers of *A. majus* (Table 1) compared to those of *L. vulgaris*. Since flowers are the only reproductive organ in *A. majus*, it is expected, according to ODT, that they would have higher levels of constitutive protection, whereas *L. vulgaris* would have lower levels, as it may still also reproduce vegetatively through adventitious roots even while flowering. In addition, on a proportional dry weight basis, *L. vulgaris* allocated less than half as much relative biomass to flowers than *A. majus*, yet had the same allocation to root tissue (Fig. 5).

In summary, our results demonstrate that the constitutive distribution and concentration of antirrhinoside in two related plant species with different reproductive strategies is generally consistent with ODT, although other explanations are possible. Our research provides a basis for further work on iridoid distribution among organs in other related plant species, as well as the potential effect of insect herbivores on plant fitness.

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zation of anti-aphrodisiacs may incur fitness costs to both females and males when natural enemies exploit them. Parasitic wasps, including egg parasitoids, often exploit the sexual communication system of their hosts during foraging (reviewed by Vinson 1984; Stowe et al. 1995; Powell 1999; Fatouros et al. 2008b).

Recently, we showed that *Trichogramma* egg parasitoids utilize the anti-aphrodisiac of one of their hosts, the gregarious large cabbage white butterfly, *Pieris brassicae* L. (Fatouros et al. 2005b; Huigens et al. 2009). Male *P. brassicae* induce female refractoriness for mating by transferring the anti-aphrodisiac, benzyl cyanide (BC), within the ejaculate (Andersson et al. 2003). Benzyl cyanide acts as a kairomone for *Trichogramma brassicae* Bezdenko and *T. evanescens* Westwood by attracting the wasps to mated *P. brassicae* females, thus facilitating phoretic transport to host oviposition sites (Fatouros et al. 2005b; Huigens et al. 2009). In addition, BC incurs another indirect cost on *P. brassicae* by triggering the release of plant compounds that arrest *T. brassicae* wasps, egg parasitoids of the butterfly (Fatouros et al. 2005a, 2008a).

Plants fed or oviposited upon by herbivores often release chemical cues (i.e., plant synomones) that attract predators and/or parasitoids, enemies of the herbivorous attackers (Hilker and Meiners 2002, 2006; Turlings and Wäckers 2004; Dicke 2009; Dicke et al. 2009). In cases known thus far, the plant's response is triggered by compounds present either in the regurgitant or in the egg-associated secretion of the herbivore (Mattiacci et al. 1995; Alborn et al. 1997; Halitschke et al. 2001; Colazza et al. 2004a; Hilker et al. 2005; Felton and Tumlinson 2008). Brussels sprouts plants respond to *P. brassicae* eggs by modifying the leaf surface, which arrests *T. brassicae* wasps three days after egg deposition when the host eggs are most suitable for parasitism (Fatouros et al. 2005b). The leaf surface changes could be of a chemical nature, but remain unknown. However, genome-wide transcriptional analysis has provided molecular evidence that confirms oviposition- and BC-induced changes in the plant (Fatouros et al. 2008a). In female *P. brassicae*, traces of BC (the male anti-aphrodisiac) were detected in the secretion from the accessory reproductive gland (ARG), which is released with eggs onto the plant surface (Fatouros et al. 2008a).

Whereas *P. brassicae* deposits egg clutches consisting of 20–50 eggs (Feltwell 1982), the closely related small cabbage white, *P. rapae* L., lays single eggs on wild and cultivated Brassicaceae (Richards 1940). Male *P. rapae* butterflies transfer methyl salicylate (MeSA) and indole with their ejaculate to the females. Mated *P. rapae* females emitting MeSA and indole were unattractive to conspecific males, thus showing that both compounds function as an anti-aphrodisiac blend (Andersson et al. 2003). The aim of this study was to investigate whether singly laid eggs of *P. rapae* induce the production of a plant synomone in Brussels sprouts that arrests *T. brassicae* wasps, and, if so, whether this plant response is triggered by male-derived compounds transferred within the ejaculate during mating, specifically, the anti-aphrodisiac compounds indole and/or methyl salicylate.

Methods and Materials

Plants and Insects Brussels sprouts plants (*Brassica oleracea* L. var. *gemmifera* cv. Cyrus) were grown in a greenhouse (18±5°C, 50–70% rh, L16:D8). *Pieris rapae* was reared on Brussels sprouts in a climate room (21±1°C, 50–70% rh, L16:D8). Virgin females were obtained by separating sexes in the pupal phase, and keeping them separately from males after eclosion until dissection. Three days after eclosion, the ARGs were dissected from females. Mated females were obtained by adding a virgin male to a virgin female one day after eclosion. As soon as a butterfly couple was observed to mate, it was isolated in a separate cage to obtain females for dissection that had mated for the first time. Two days after mating, the ARG were dissected from females.

Trichogramma brassicae (Hymenoptera: Trichogrammatidae) was reared in irradiated eggs of the moth *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), received from Koppert B.V., The Netherlands, at 25±1°C, 50–70% RH, L16:D8 in a climate chamber. *Ephestia kuehniella* eggs were glued on paper cards and offered to the wasps in glass vials for parasitization. *Trichogramma* wasps are known to be capable of developing successfully in *Ephestia* eggs (Brower 1983). Only mated, 2-5-d-old, oviposition-experienced female wasps were used for the experiments. An oviposition experience was given for a period of 18 h prior to the experiment with ≤3-d-old *P. rapae* eggs deposited on Brussels sprouts leaves. Eggs older than 3 d are unsuitable for parasitization because of sclerotization of the caterpillar's head capsule.

Preparation of ARG Homogenates To obtain samples for bioassays, ARGs were dissected from 3 gravid or virgin *P. rapae* females (3-d-old) in phosphate buffered saline (PBS; pH 7.2), transferred to a vial with 100 µl PBS, and homogenized. Then, after addition of another 100 µl of PBS, the homogenate was centrifuged, and 100 µl of the supernatant were applied with a brush to the edge of a cabbage leaf as described below (an equivalent of 1.5 extracted glands per leaf). For chemical analysis, 1 ARG of either a mated or a virgin *P. rapae* female (3-d-old) was dissected and transferred to a vial containing 50 µl of dichloromethane (DCM) with ethyl salicylate (EtSA) as internal standard (0.25 ng/µl). In total, 7 samples of each gland type were analyzed by GC-MS.

Plant Treatments For bioassays with egg-infested plants, test plants were placed into a cage with more than 100 *P. rapae* adults to allow deposition of eggs, wing scales, and host odors onto the plants. Plants were exposed for about 1–2 h to the butterflies, with a maximum of 20 eggs deposited per leaf. Up to 20 *P. rapae* eggs have been observed on a single *Brassica oleracea* plant in nature (N.E. Fatouros, personal observations). After this exposure time, the egg-infested plants were tested immediately or were kept in a climate chamber (21±2°C, 70% rh, L16:D8) either overnight (24 h) or 48 to 72 h after the day of egg deposition. Thus, the period during which eggs or butterfly deposits could affect the cabbage plant was in total 6, 24, 48, 72, or 96 h. Control plants were grown under the same conditions as treated plants, but were never in contact with *P. rapae* or other insects.

For bioassays with ARG homogenate-treated plants, a sample of ARG homogenate of mated *P. rapae* females was applied to the edge of a Brussels sprouts leaf in a stretch of about 2 cm on the abaxial leaf side. As a control, a sample of ARG homogenate of virgin *P. rapae* females was applied to the plants in the same way. After treatment, all plants were kept for either 24 or 72 h in a climate chamber (21±1°C, 50–70% RH, L16:D8).

For bioassays with ARG homogenate and indole treated plants, 30 µl of methanolic indole solution (10 ng indole (Sigma-Aldrich) in 100 µl MeOH) were added to 170 µl of ARG homogenate from virgin females, and 100 µl of this mixture were applied to a leaf as above (i.e., 1.5 ng indole, plus ARG homogenate from virgin females equivalents 1.5 ARGs from mated females). Test leaf squares were cut from the plant 72 h after application of the mixture. Control leaf squares were obtained from leaves treated with ARG homogenate of virgin females in PBS and MeOH.

For bioassays with indole treated plants, 100 µl of 0.005 or 0.5 ng indole/µl MeOH solutions were applied to leaves as described above. Control plants were treated with 100 µl MeOH only. Leaf squares adjacent to the indole treatment were tested against leaf squares adjacent to the solvent treatment in a two-choice bioassay.

Two-chamber Olfactometer Bioassays The experiments were carried out in a two-chamber olfactometer described in detail by Fatouros et al. (2005a). Time spent by wasps in one of the two odor fields was measured for 300 s. The 4th or 5th leaf from the top of a plant was excised, and kept with its petiole in a vial with water during the bioassay. A total of 10 *T. brassicae* wasps were tested per day per plant. In total, 50 wasps per treatment were tested, and 5 plants per treatment were used. To avoid biased results due to positional preferences of the parasitoids, the olfactometer was rotated 180° after every third insect tested. The response of *T. brassicae* was tested to odors of plants infested with *P. rapae* eggs in the following two-choice combinations: a) plants with eggs 24 h after deposition vs. clean air and, b) plants with eggs 72 h after egg deposition vs. clean air. All two-chamber olfactometer bioassays were statistically analyzed using *Wilcoxon matched pairs test* by using SPSS for Windows 15.0 Software.

Two-choice Contact Bioassays All contact bioassays were conducted with egg-free or untreated leaf squares (in Fatouros et al. 2005a, 2008a, denoted as 'locally induced') cut close to a treated leaf part, i.e., single eggs, applied ARG homogenate and/or indole/MeOH solution. A female wasp was released in a glass Petri dish (5.5 cm diam) halfway between a test, and control leaf squares (1.5 cm^2) directly cut from the plants immediately prior to the bioassay. The total time spent (i.e., residence time) on each of the leaf squares was observed for a period of 300 s by using The Observer software v. 4.0 (Noldus Information Technology, Wageningen, The Netherlands). A detailed description of the bioassay method is given elsewhere (Fatouros et al. 2005a). Test and control squares were taken from leaves of corresponding size and position on the plants. In total, 50 wasps per treatment were tested, and 5 plants per treatment were used. A maximum of 10 wasps per experimental day were tested. Leaf squares were renewed and repositioned randomly after every third wasp tested. All two-choice contact bioassays were analyzed using *Wilcoxon matched pairs test* by using SPSS 15.0 for Windows Software.

Chemical Analysis ARG extracts were analyzed by coupled gas chromatography—mass spectrometry (GC-MS) using a gas chromatograph (Agilent 7890A) equipped with a 30-m Zebron ZB-5 ms column (0.25 mm i.d., 0.25-µm film thickness; Phenomenex, Torrance, CA, USA) and an inert mass selective detector (model 5975C with triple axis detector, Agilent). A 5 m Guardian™ pre-column (deactivated fused silica tubing without stationary phase; Phenomenex, Torrance, USA), was permanently attached to the analytical column. The GC was programmed from 45°C for 1 min, to 200°C at 10°C min^{-1}, then to 280°C at 30°C min^{-1}, and held 3.5 min. The sample volume (1 µl) was injected in splitless mode. The injection port and interface temperature were 250°C and 280°C, respectively, and the helium inlet pressure was controlled electronically to achieve a constant column flow of 1.0 ml min^{-1}. A solvent delay was set to 4 min. The ionization potential was set at 70 eV, and scanning was performed from 33 to 200 atomic mass units. Identification of MeSA, indole and the internal standard EtSA was based on the injection of authentic reference standards (>98% purity, Sigma-Aldrich). Quantification of MeSA and indole was based on comparison with the internal standard. A calibration

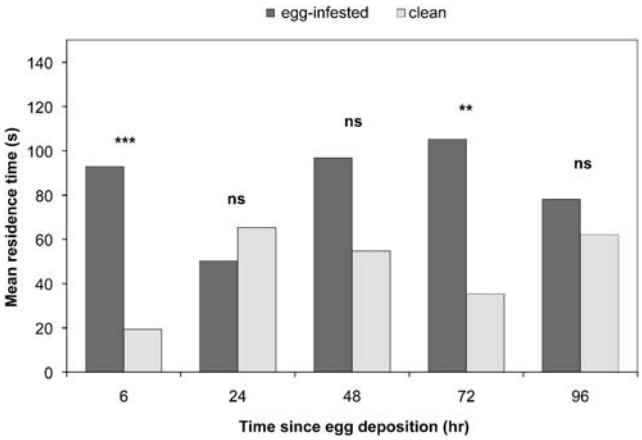

Fig. 1 Mean residence time of *Trichogramma brassicae* females on Brussels sprouts leaves infested with *Pieris rapae* eggs (egg-infested) tested against leaves of uninfested plants (clean). Egg-free leaf squares adjacent to eggs that had been deposited 6-96 h prior to the bioassay (dark grey columns) were simultaneously offered against leaf squares from uninfested plants (light grey columns) in a two-choice contact bioassay in a Petri dish. Number of tested females per treatment, N=50. Abbreviations: ns—not significant; **P<0.01; ***P<0.001 (*Wilcoxon matched pairs test*)

series of MeSA, indole, and EtSA, injected from a concentration of 0.05–50 ng/µl DCM, showed similar linear response factors of all three compounds within this concentration range (data not shown). Differences in quantities of anti-aphrodisiac compounds were analyzed using *Mann-Whitney U test* by using SPSS 15.0 for Windows Software.

Results

Olfactory Response of Trichogramma to Leaves Infested with P. rapae Eggs Trichogramma brassicae did not discriminate between volatiles from an egg-laden leaf and clean air (24 h: P=0.426, 72 h: P=0.903, *Wilcoxon matched pairs test*).

Arrestment of Trichogramma by Leaves Infested with P. rapae Eggs Leaf squares from plants, on which eggs had been deposited 6 h prior to the assay arrested *T. brassicae* when tested vs. leaf squares from uninfested plants (Fig. 1, P<0.001, *Wilcoxon matched pairs test*). However, leaf squares from plants on which eggs had been deposited 24 h prior to the assay did not arrest the wasps (Fig. 1, P=0.494, *Wilcoxon matched pairs test*). The wasps tended to prefer leaf squares cut from an egg-carrying leaf on which eggs had been deposited 48 h prior to the assay when tested vs. leaf squares from uninfested plants (Fig. 1a, P=0.058, *Wilcoxon matched pairs test*). Wasps stayed longer on leaf squares of plants with eggs deposited 72 h prior to the bioassay (Fig. 1, P=0.004, *Wilcoxon matched pairs test*). Four days after egg deposition, wasps did not discriminate between leaf squares of egg-infested plants when tested vs. leaf squares of uninfested plants (Fig. 1a, P=0.427, *Wilcoxon-matched pairs test*).

Arrestment of Trichogramma by Leaves Treated with ARG Untreated leaf squares obtained from leaves treated with ARG homogenates of mated females were tested against

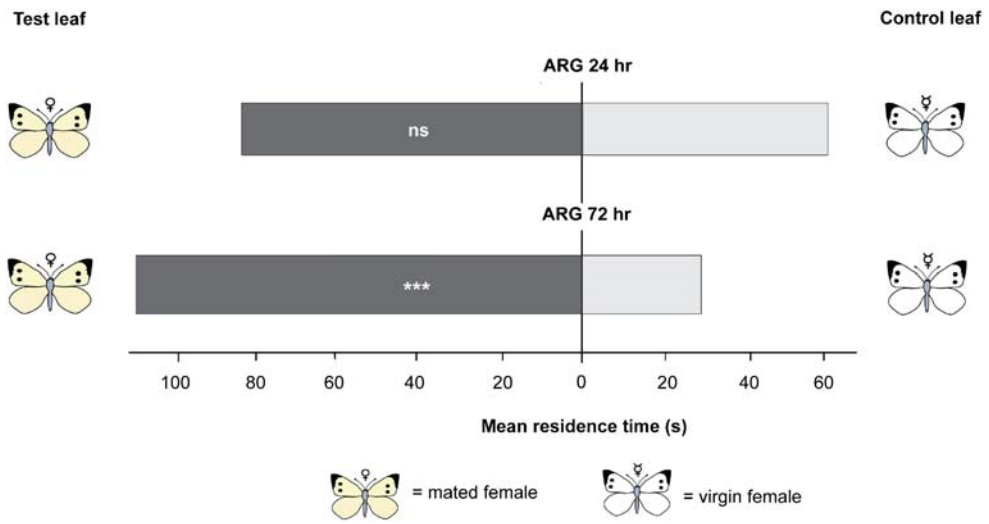

Fig. 2 Mean residence time of *Trichogramma brassicae* females on Brussels sprouts leaves treated with accessory reproductive gland (ARG) homogenate of *Pieris rapae* females. Leaves were treated either with ARG homogenate from virgin females (light grey bars) or ARG homogenate from mated females (dark grey bars) a) 24 h or b) 72 h prior to the bioassay. Untreated leaf squares, adjacent to a site on the same leaf that was treated with ARG homogenate, were simultaneously offered to the wasps in a two-choice contact bioassay in a Petri dish. Number of tested females wasps per bioassay combination, N=50. Abbreviations: ARG—accessory reproductive gland; ns—not significant; ***P<0.001 (*Wilcoxon matched pairs test*)

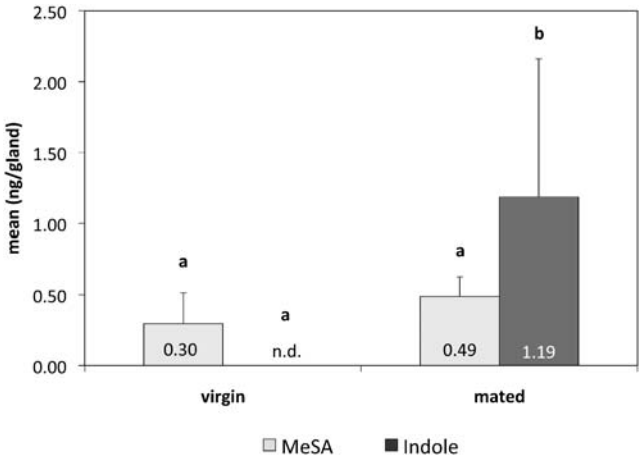

Fig. 3 Quantities of anti-aphrodisiac compounds detected by GC-MS in accessory reproductive gland (ARG) extracts from virgin or mated *Pieris rapae* females. Mean (± s.d.) amount of methyl salicylate (grey columns) and indole (dark grey column) per gland is shown. Number of dissected ARG, N=7 of each treatment. Different letters indicate significant differences in quantity within the same compound between the ARG extracts from virgin and mated females (*Mann-Whitney U test*). Abbreviations: ARG—accessory reproductive gland; MeSA—methyl salicylate; n.d.—not detected

untreated leaf squares from leaves treated with ARG homogenate of virgin females 24 h after treatment. The wasps did not discriminate between the two leaf squares cut from the vicinity of leaf area treated with the ARG homogenate (Fig. 2, P=0.184, *Wilcoxon matched pairs test*). However, 72 h after ARG application, the wasps spent significantly more time on leaf squares taken from plants treated with ARG extract of mated *P. rapae* females than on leaf squares from plants treated with ARG extract of virgin *P. rapae* females (Fig. 2, P<0.001, *Wilcoxon matched pairs test*).

Chemical Composition of ARG Extracts Chemical analysis of ARG secretion of mated females revealed the presence of the anti-aphrodisiac compounds (Andersson et al. 2003), indole and MeSA. In ARG extracts obtained from females 2 days after mating, 1.2±0.97 ng indole and 0.49±0.14 MeSA were detected (Fig. 3). In ARG extracts of virgin females, 0.30±0.22 ng MeSA / ARG was detected, whereas indole was lacking (Fig. 3, P<0.001, *Mann-Whitney U test*).

Arrestment of Trichogramma by Leaves Treated with Indole Untreated leaf squares taken from leaves treated with ARG homogenate from virgin females supplemented with 1.5 ng indole arrested the wasps 72 h after application when tested against untreated leaf squares obtained from leaves treated only with ARG homogenate from virgin females (Fig. 4, P<0.001, *Wilcoxon matched pairs test*). Leaf squares of plants treated with 50 ng indole alone did not arrest the wasps 24 h after treatment (Fig. 4, P=0.134, *Wilcoxon matched pairs test*). Leaf squares of leaves treated with indole 72 h after treatment arrested *T.*

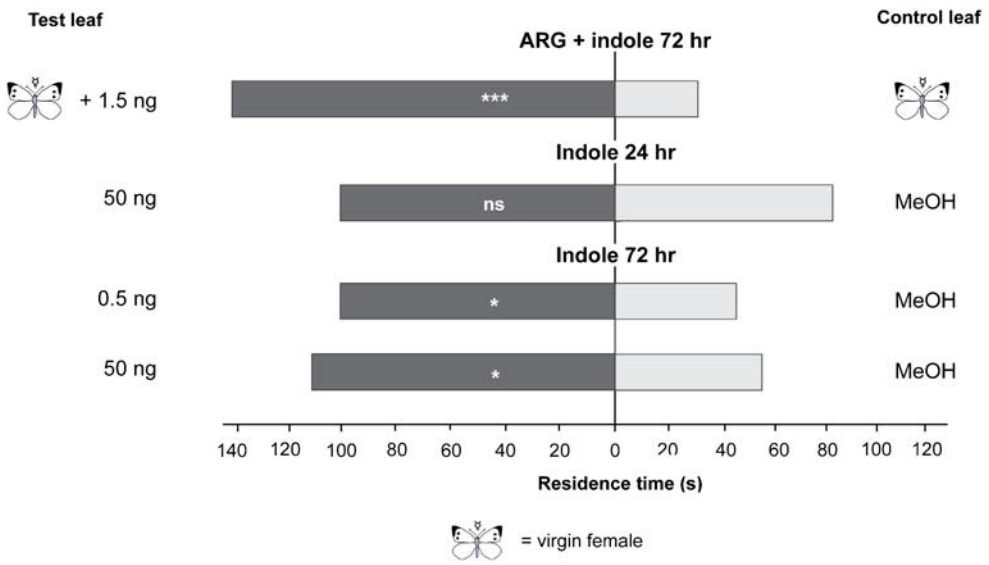

Fig. 4 Mean residence time of *Trichogramma brassicae* females on Brussels sprouts leaves treated with Accessory Reproductive Gland (ARG) homogenate of virgin females and indole or treated with indole alone (amount shown in ng). Test leaves were treated either with ARG extracts from virgin *P. rapae* females and indole or indole alone (dark grey bars) and tested against control leaves treated with ARG extracts from virgin *Pieris rapae* females or methanol only (light grey bars) a) 24 h or b) 72 h prior to the bioassay. Untreated leaf area, adjacent to a site on the same leaf that was treated with ARG homogenate and/or indole was simultaneously offered against leaf squares taken from ARG-homogenate or methanol-treated leaves in a two-choice contact bioassay in a Petri dish. Female wasps per bioassay combination, N= 50. Abbreviations: MeOH—methanol; ARG—accessory reproductive gland; ns—not significant; *P<0.05; ***P<0.001 (*Wilcoxon matched pairs test*)

brassicae wasps when tested against leaf squares of plants treated with solvent only at both tested concentrations (Fig. 4, 0.5 ng: P=0.028; 50 ng: P=0.036, *Wilcoxon matched pairs test*).

Discussion

Our data show that egg deposition of the small cabbage white butterfly, *P. rapae*, induces Brussels sprouts plants such that *T. brassicae* wasps are arrested 72 h after oviposition, whereas plants with eggs deposited 24 or 48 h before testing did not elicit this behavior (Fig. 1). Thus, we demonstrated that butterfly eggs deposited singly and spaced can trigger an indirect plant response similar to that induced by egg batches of herbivorous insects (Meiners and Hilker 2000; Hilker et al. 2002; Colazza et al. 2004a; Fatouros et al. 2005a). Most butterfly species deposit their eggs singly, and these often are cryptically colored. It is assumed that natural selection by predators and parasitoids favors this strategy. Singly laid eggs probably have lower amounts of toxins relative to the often aposematically colored eggs laid in clutches (Stamp 1980). We expected that the induced response of Brussels sprouts plants to eggs of *P. rapae* would be less detectable by *Trichogramma* wasps than the response to *P. brassicae* eggs. This contingency would have been consistent with the findings of Little et al. (2007) who showed that deposition of *P. rapae* eggs on *Arabidopsis thaliana* caused a much weaker transcriptional response of defense- and stress-related genes than did *P. brassicae* egg clutches.

When an extract from accessory reproductive glands (ARGs) of mated female butterflies was applied to plants, the wasps were arrested on treated plants 72 h after ARG treatment, but not after 24 h. This shows that the ARG secretion itself triggered the plant response and not the eggs *per se*. One known function of the ARG is the secretion of adhesive polymers to attach eggs onto the leaf surface (Gillott 2002). The ARG extract from virgin females applied onto the plant arrested the wasps neither 24 nor 72 h after application (Fig. 2). Evidently, compounds derived from the seminal fluid of the male butterfly have changed the composition of the female's ARG, ultimately playing a part in triggering the plant response.

Our evidence indicates that the male anti-aphrodisiac compound, indole, reaches the ARG of a mated *P. rapae* female. No indole was found in the ARG of virgin females (Fig. 3). Subsequently, *T. brassicae* wasps had a significant preference for leaves treated with the ARG of virgin females plus indole over leaves treated with ARG extract from virgin females. The parasitoids' response to leaves treated with indole alone (without ARG extract) 72 h after application was lower but still significant (Fig. 4). Thus, a single compound, which is part of the two-component anti-aphrodisiac blend of *P. rapae* males and transferred with the male ejaculate, is likely to trigger this plant defense in response to egg deposition. Earlier, Doss et al. (2000) showed that esters of long-chain diols (the so-called bruchins) elicit formation of callus tissue in pea pods when certain species of Bruchidae (Coleoptera) oviposit in pods. Our discoveries that anti-aphrodisiac compounds in *Pieris* spp. act similarly as elicitors add to the known instance of this type of effect (Fatouros et al. 2008a).

Interestingly, the anti-aphrodisiac compounds of *Pieris* spp. (BC, MeSA and indole) are ubiquitous phytochemicals produced, for example, as part of herbivore-induced plant volatiles (HIPV) from several plant species; e.g., Lima bean (Dicke et al. 1990; De Boer et al. 2004), tomato (Ament et al. 2004), and maize (D'Alessandro and Turlings 2005). Cultivated *Brassica* species and *Arabidopsis thaliana* emit BC, MeSA, and indole after feeding by *Pieris* caterpillars or after the application of their regurgitant (Geervliet et al. 1997; van Poecke et al. 2001; Smid et al. 2002; Fatouros et al. 2005c). The biological relevance of these HIPV components has been proven for many herbivorous and carnivorous species. Methyl salicylate and indole derivates repel parasitoids of lepidopteran larvae (D'Alessandro et al. 2006; Snoeren 2009) and ovipositing lepidopterans (De Vos et al. 2008; Ulland et al. 2008), whereas predatory arthropods are attracted (De Boer and Dicke 2004; De Boer et al. 2004; James and Price 2004; Ishiwari et al. 2007). In several plant species, the two aromatic compounds, MeSA and indole, are formed via the shikimic acid pathway (Paré and Tumlinson 1997), whereas the nitrile (BC) is a myrosinase-catalyzed hydrolysis product of glucosinolates in Brassicaceae (reviewed by Grubb and Abel 2006; Hopkins et al. 2009). However, in *Pieris*, BC, MeSA, and indole are not plant-derived; rather, males utilize the amino acids phenylalanine and tryptophan as precursors for these anti-aphrodisiac compounds (Andersson et al. 2000, 2003).

The waxy leaf surface of Brussels sprouts plants shows no apparent damage below or around eggs of *Pieris* spp. (see Chapman and Bernays 1989 and references therein; N. E. Fatouros, personal observations). All other studies of indirect plant responses against herbivore oviposition involved wounding by the ovipositing female, leading to emission of volatiles attractive to egg parasitoids (Hilker and Meiners 2006). For examples, eggs of an elm leaf beetle (*Xanthogaleruca luteola*) and a pine sawfly (*Diprion pini)* are laid on the plant surface or into tissue damaged by the egg-laying female prior to oviposition; the oviposition-associated leaf damage in these species may

provide improved attachment to the plant or protection of the eggs by the plant tissue (Meiners and Hilker 1997, 2000; Hilker et al. 2002). Eggs laid by the heteropteran, *Nezara viridula*, induced a volatile synomone for the egg parasitoid *Trissolcus basalis* in two legume species (*Vicia faba* and *Phaseolus vulgaris*), but only when oviposition and host feeding occurred together on the same plant (Colazza et al. 2004a, 2004b). Thus, we assumed that wounding of the leaf surface during or immediately preceding the egg deposition is required to induce volatile long-range cues that attract the egg parasitoids (Fatouros et al. 2005b). Yet, evidence is accumulating that in other *Brassica* species that carry less surface waxes, *Pieris* egg deposition induces volatile emissions attractive to *Trichogramma* wasps (N. E. Fatouros, unpublished data). Differences in the physical structure and/or chemical composition of the plant cuticle could play an important role in the adsorption and/or absorption of molecules into leaf cells, thus triggering certain pathways involved in plant defense against herbivores. Leaf wax microstructure and chemical composition affect herbivores, and also indirectly influence their predators and parasitoids (Eigenbrode and Espelie 1995). For example, an increased surface wax layer has been found to confer higher resistance to herbivores (see Müller and Riederer 2005 and references therein).

Egg-free leaf parts of plants on which eggs had been deposited elsewhere 6 h before testing did arrest *T. brassicae* wasps. This arrestment is probably due to host residues, such as wing scales, as Fatouros et al. (2005a) showed that *T. brassicae* responded to *P. brassicae* egg-free leaves contaminated with butterfly deposits for 24 h; however, the response disappeared after 72 h. Scales and other chemical traces of host insects frequently are used as kairomones by egg parasitoids (reviewed by Fatouros et al. 2008b). The wings of *Pieris* butterflies emit chemical cues, some of which have pheromonal activity (Arsene et al. 2002; Andersson et al. 2007). The aphrodisiacs from male scent scales of *P. rapae* and *P. brassicae* (two macrolides) recently have been identified (Yildizhan et al. 2009). Whether these macrolides arrest wasps on leaves with eggs younger than 24 h remains to be tested.

Sexual signals produced that attract mates (or that repel the competing sex as in the case of anti-aphrodisiac pheromones) potentially incur trade-offs associated with their use as kairomones by natural enemies (Stowe et al. 1995; Zuk and Kolluru 1998; Fatouros et al. 2008b . It is assumed that female sex pheromones are rarely exploited because of their high specificity, low intensity, and emission in enemy-free space and time (Zuk and Kolluru 1998). However, an increasing number of studies have shown that egg parasitoids in particular exploit adult host sex pheromones (Fatouros et al. 2008b). In contrast to sex pheromones, the release of anti-aphrodisiacs is unlikely to be under female control (Andersson et al. 2004). There should be enough time for egg parasitoids to detect and approach a mated female, in contrast to a virgin, to successfully parasitize fertilized host eggs. By triggering parasitoid-attracting plant cues, the anti-aphrodisiac compounds incur an additional cost of mating, namely, increased egg mortality and lower butterfly fitness. We postulate, therefore, that the evolution of anti-aphrodisiac compounds transferred during copulation by the males is selected against by *Trichogramma* wasps.

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we trapped 86,471 subcortical insects (143 species) in baited and unbaited Lindgren funnel traps, and 500 beetles (44 species) in baited and unbaited pitfall traps. We report 23 new state collection records of subcortical insects from Minnesota. Trap catches of subcortical insects were greatest in the wind-disturbed areas 2 years after the event, and declined thereafter. Similar trends were observed for subcortical insects in the burned areas. Both wind-disturbance and burning increased the subcortical insect species richness and diversity on the landscape. The subcortical insect species compositions of the salvaged and burned forest areas differed from those of the undisturbed and wind-disturbed areas. Trap catches of subcortical insects in response to semiochemical treatments also varied with year of sampling and land-area treatment. The greatest diversity of subcortical beetle species was in traps baited with attractants for the scolytids, *Dendroctonus valens* [(+)-α-pinene and (−)-β-pinene] and *Dryocoetes* spp. [*exo*-brevicomin and (−)-α-pinene], perhaps reflecting the generic nature of the baits. The most distinct species compositions were collected in response to the woodborer and *Dendroctonus simplex* baits, whereas the species compositions in traps with the *D. valens* and *Dryocoetes* spp. baits, and the unbaited funnel trap were the most similar. The variation in trap catch with time and across landscapes suggests that the responses of subcortical insects to semiochemicals are more complex than previously appreciated.

Keywords Anthribidae · Bark beetles · *Betula papyrifera* · Biodiversity · Buprestidae · Cerambycidae · Cleridae · Coleoptera · Curculionidae · *Dendroctonus rufipennis* · *Dendroctonus simplex* · *Dendroctonus valens* · *Dryocoetes autographus* · Histeridae · Host attractants · *Hylurgops rugipennis pinifex* · Ipsdienol · Ipsenol · *Ips grandicollis* · *Ips perroti* · *Ips perturbatus* · *Ips pini* · Kairomones · Minnesota · Natural disturbances · Nemonychidae · Pheromones · *Picea glauca* · Pinenes · *Pinus banksiana* · *Populus tremuloides* · Predators · Salpingidae · Scolytidae · *Sirex behrensii* · Siricidae · Semiochemicals · Tenebrionidae · Wind-disturbance · Woodborers

Introduction

On 4 July 1999, a catastrophic storm with wind derechos (straight-line winds with multiple downbursts) of over 145 km hr^{-1} in the Superior National Forest (in northeastern Minnesota, USA) resulted in the wind-throw of over 193,000 ha of forestland (USDA Forest Service 2000). This long-duration and extensive wind storm originated in North Dakota, lasted less than 24 h, and traveled over 2,100 km from northern Minnesota into southern Ontario and Quebec, and northern New Hampshire, Vermont, and Maine (NOAA 2005). In Minnesota, over 85% of the most severe damage (areas with 67–100% tree mortality) occurred in the Boundary Waters Canoe Area Wilderness (BWCAW), where no forest management activity is permitted (see maps in Gandhi et al. 2005). Some damage also occurred along the Gunflint Trail Corridor, which is a heavily transited entry into the BWCAW and an access route to numerous vacation homes and service structures. Such a catastrophic storm was unique both in its extent (193,000 ha), scale of damage (>70% tree mortality in most areas), and long-interval duration (once every 1,000 yr) (Frelich and Lorimer 1991; USDA Forest Service 2000). With dead and downed trees (fuel loads) exceeding 16,000 kg/ha in some of the wind-disturbed areas (Gilmore et al. 2003), there was a strong potential for the occurrence of other natural disturbances such as wildfire, and insect and disease outbreaks. In 1999–2002, in response to these threats to remaining forest stands in this area, the USDA Forest Service reduced the fuel load along the Gunflint Trail Corridor through: (1) salvage-logging (1,100 ha), and (2) prescribed-burning (860 ha).

Subcortical (phloeophagous, xylophagous, and rhizophagous) forest insects are the first to colonize, reproduce, and increase populations in wind-disturbed forested landscapes (Connola et al. 1956; Gardiner 1975; Ayres et al. 1999). These early-successional species colonize the phloem and outer xylem of the tree tissue before it has desiccated completely (Gandhi et al. 2007). In wind-damaged forests, habitats for these species typically include uprooted trees; fallen branches and tree parts (slash); partially damaged, moribund, and living standing trees; and undamaged residual trees [collectively known as coarse-woody debris (CWD)]. Weakened, moribund, and undamaged residual standing trees, which die as a consequence of the aftermath of the storm, provide additional subcortical insect habitat for several years after the wind disturbance. The condition of CWD changes with time due to fermentation of xylem, decay of phloem, bark slough-off, breakage of stems and branches, and accumulation of additional CWD on the forest floor. The diversity and abundance of CWD created by a wind-disturbance provides excellent habitat for subcortical insects, and there is a temporal progression of species that exploit CWD with fresh phloem through those that exploit CWD with drying or decaying xylem. A large number of predators and parasites also are associated with subcortical herbivorous insects (Dahlsten 1970; Ryall 2003). Thus, as the amounts and characteristics of CWD change over time (Ryall and Smith 2005; Wilson and McComb 2005), there will be changes and turnover of associated subcortical insect communities (Weiss 1920; Blackman and Stage 1924; Savely 1939; Wallace 1953).

Historically, through rearing and collecting, workers have described the guilds of subcortical insects associated

with trees such as basswood, *Tilia americana* L. (Townsend 1886); tamarack, *Larix laricina* (Du Roi) K. Koch (Blackman and Stage 1918); shagbark hickory, *Carya ovata* (P. Mill.) K. Koch (Blackman and Stage 1924); shortleaf pine, *Pinus echinata* Mill, loblolly pine, *Pinus taeda* L., white oak, *Quercus alba* L., northern red oak, *Q. rubra* L., southern red oak, *Q. falcata* Michx., black oak, *Q. velutina* Lam., and post oak, *Q. stellata* Wangenh. (Savely 1939); and Scots pine, *Pinus sylvestris* L. (Wallace 1953). More recently, such community level taxonomic characterizations in North America have included insects associated with aspen, *Populus* spp. (Hammond 1997; Hammond et al. 2001); butternut, *Juglans cinerea* L. (Katovich and Ostry 1998); Douglas-fir, *Pseudotsuga menziesii* (Mirbel) Franco, hemlock, *Tsuga* spp. (Endlicher) Carrière, and lodgepole pine, *Pinus contorta latifolia* (Engelmann) Critchfield (Deyrup and Gara 1978); elms, *Ulmus* spp. L. (Hajek and Dahlsten 1985); loblolly and shortleaf pines, *Pinus taeda* and *P. echinata* (Dixon and Payne 1979; Berisford 1980); Monterey pine, *Pinus radiata* D. Don (Ohmart 1981; Ohmart and Voigt 1982); ponderosa pine, *Pinus ponderosa* Laws. (Dahlsten 1970; Stephen and Dahlsten 1976a, b); red pine, *Pinus resinosa* Ait. (Aukema and Raffa 2000, 2005; Aukema et al. 2000a, b, 2004, 2005; Erbilgin and Raffa 2001; Erbilgin et al. 2003); sugar pine, *Pinus lambertiana* Dougl. (Dahlsten and Stephen 1974); and white spruce, *Picea glauca* (Moench) Voss (Whitmore 1982; Gara et al. 1995; Haberkern et al. 2002). In Europe, community level work on subcortical forest insects also has been conducted in beech, *Fagus sylvatica* L. (Schiegg 2001); Norway spruce, *Picea abies* (L.) Karst, and *Pinus sylvestris* (Kaila et al. 1997); and subalpine spruce forests (Wermelinger et al. 2002, 2003; Joensuu et al. 2008).

Semiochemicals are being used increasingly to assess forest insect conservation biology and biodiversity (Svensson et al. 2004; Tolasch et al. 2007; Svensson and Larsson 2008; Larsson and Svensson 2009), and flight traps (with or without lures) are replacing hand collecting or rearing to assess the biodiversity and community ecology of subcortical insects (Ranius and Jansson 2002). Flight and pitfall traps baited with semiochemicals, such as aggregation pheromones and kairomones (Wood 1982a; Chénier and Philogène 1989), also can be used to monitor the diversity and population abundance of early successional subcortical insects following ecological disturbances (Werner 2002). In fact, semiochemicals have been a research and management tool for bark beetles for over 40 years (Wood et al. 1968), especially in western North American forests where these beetles can be devastating (Bedard and Wood 1981; Negrón et al. 2008). In many cases, semiochemicals were first identified from western North American bark beetle species (Silverstein et al. 1966, 1967; Wood et al. 1967; Furniss et al. 1972; Baker et al. 1977; Birch et al. 1980; Bowers et al. 1991; Hobson et al. 1993; Borden et al. 1996; Camacho et al. 1998; Holsten et al. 2000), and baits in other regions of the continent often are based on these research results. In addition to targeting specific species, semiochemicals also can be used to assess the diversity of predators and commensals that exploit the attractants of subcortical insects as host-finding kairomones (Dixon and Payne 1980; Phillips et al. 1988; Chénier and Philogène 1989; Seybold et al. 1992; Aukema and Raffa 2000, 2005; Erbilgin and Raffa 2001).

Presently, little is known about semiochemicals of subcortical insects and their associates in the sub-boreal forests of north-central North America. Studies on the semiochemicals of forest insects across different stand types within a landscape (such as the BWCAW) or over multiple field seasons are rare. Most semiochemical studies have been conducted over only one or two summers, either within relatively homogenous stands (e.g., single species pine stands of similar ages) (Miller and Borden 2003) or concurrently during one season across wide geographic areas (Miller et al. 1997). Finally, few studies have explored the full range of subcortical forest insects by using traps baited with semiochemicals. Most semiochemical studies have focused on either single subcortical species (e.g., Bedard et al. 1980; Borden et al. 1996); their associated predators (e.g., Seybold et al. 1992; Erbilgin and Raffa 2001); or on a small subset of subcortical insect families (e.g., Phillips et al. 1988; Chénier and Philogène 1989; Miller and Rabaglia 2009).

We studied the responses of phloeophagous, xylophagous, and rhizophagous insects and their associates to semiochemical treatments in the context of an ecological disturbance, the severe wind-disturbance event and subsequent management activities in a northeastern Minnesota sub-boreal forest. We targeted Buprestidae, Cerambycidae, and Scolytidae because they are the most economically important subcortical taxa, are early colonizers of CWD, and are crucial to CWD breakdown and nutrient cycling (Craighead et al. 1927; Craighead 1950; Furniss and Carolin 1977; Jacobs 2004). Among the primary subcortical insects, these taxa are the most species-rich and numerically abundant in sub-boreal forests (Dodge 1938; Bright 1976, 1987; Downie and Arnett 1996; Yanega 1996). Our specific research objectives were: 1) to test the efficacy of various known semiochemical treatments to monitor subcortical insects in a sub-boreal forest; and 2) to use trap-catch to document population responses of subcortical insects to a severe wind-disturbance event (>70% tree mortality) and fuel-reduction (salvaging and burning) treatments. We studied these subcortical insects for 4 years because we hypothesized that populations and assemblages of subcortical beetles would track the changing conditions of the CWD in the disturbed stands (Jacobs 2004).

Methods and Materials

Study Sites Subcortical insects and their associates were sampled from 28 sites in the BWCAW, and along the Gunflint Trail Corridor in the Superior National Forest in Cook County, MN, USA (Gandhi 2005; Gandhi et al. 2008). Mean annual temperature of the region is 1–2°C (Baker and Strub 1965), and mean annual precipitation is 65–75 cm (Baker and Kuehnast 1978). This area is located in the Laurentian mixed forest ecological province, northern Superior uplands section, and border lakes subsection of Minnesota (Minnesota Department of Natural Resources 1999). Soils along the Gunflint Trail have been classified primarily as the soil orders Inceptisols and Entisols (Anderson et al. 2001); major cover types in this area are aspen/birch/conifer (ABC) and jack pine, *Pinus banksiana* Lamb. (JP) (Table 1). In general, the ABC sites are on deeper, moist, well-drained till, outwash and lacustrine deposits, and loamy Entisols [Land Type Associations (LTA) 14, 22, 23, 37], whereas the JP sites are on shallow, well-drained till and outwash deposits, and sandy Inceptisols (LTA 21, 23) (USDA Forest Service 2000). The ABC forests originated after clear-cutting, whereas the JP forests originated after a wildfire (M. Theimer, personal communication, USDA Forest Service, Superior National Forest). Forest overstory of the ABC type is composed of trembling aspen, *Populus tremuloides* Michx., paper birch, *Betula papyrifera* Marsh., eastern white pine, *P. strobus* L., red pine, *P. resinosa*, black spruce, *Picea mariana* (P. Mill.) B. S.P., white spruce, *P. glauca*, balsam fir, *Abies balsamea* (L.) P. Mill., northern white cedar, *Thuja occidentalis* L., and tamarack, *L. laricina*. Forest overstory of the JP type is composed of jack pine, *P. banksiana*, and *B. papyrifera*. Adjoining and low-lying areas often contained *L. laricina* and *T. occidentalis* in riparian habitats. Forest understory of the two cover types is characterized by *Corylus cornuta* Marsh., *Vaccinium* spp., *P. tremuloides*, *A. balsamea*, and *P. glauca* (Gilmore et al. 2002, 2005; Johnson 2004).

Table 1 Attributes of tree species in two forest cover types in the study plots in the Superior National Forest, Cook Co., MN, USA

Stand Attributes[a]	Aspen/ Birch/ Conifer[b]	Jack Pine[b]
Soil pH	5.31±0.05	5.02±0.07
Mean Age (years)	71.33±3.62	75.29±1.64
Total Basal Area (m^2/ha)	22.10±8.24	25.37±6.70
Basal Area (m^2/ha) by Species		
Aspen, *Populus tremuloides* Michx.	10.95±2.98	2.33±1.50
Jack Pine, *Pinus banksiana* Lamb.	2.50±1.85	18.17±2.60
Balsam Fir, *Abies balsamea* (L.) P. Mill.	2.18±0.4	0.67±0.33
Paper Birch, *Betula papyrifera* Marsh.	5.12±2.23	0.83±0.37
Black Spruce, *Picea mariana* (P. Mill.) B.S.P.	1.35±0.78	3.37±1.90

[a] From Gilmore et al. (2002, 2005, unpublished data) and Johnson (2004)

[b] Data are means ± standard error

Experimental Design

Land-Area Treatments During the summer of 2000, we sampled from study sites in the ABC and JP forest cover types, whereas during the summers of 2001–2003, only JP sites were sampled for logistical reasons and because results in 2000 suggested that JP sites had the greatest subcortical insect diversity. In 2000, mature *P. tremuloides* and *Pinus banksiana* trees on these sites had mean ages of 71 years and 73 years, respectively. In 2000, we sampled two sites (replicates) for each of three treatment types [(1) undisturbed forests (control); (2) severely wind-disturbed forests (>70% tree mortality); and (3) wind-disturbed-salvage-logged (salvaged) forests] in each of the two (ABC and JP) cover types for a total of 12 sites. In 2001–2003, we sampled four sites (replicates) for each of the four treatment types [(1) undisturbed, (2) severely wind-disturbed, (3) salvaged, and (4) wind-disturbed-prescribed-burned (burned) forests] in the JP cover type for a total of 16 sites (Gandhi 2005; Gandhi et al. 2008). The wind-disturbed sites were salvaged between the summer of 1999 and the spring of 2000, and burned between the fall of 2000 and the spring of 2001 (Gandhi 2005; Gandhi et al. 2008). The sites were 2–3 ha in size, and located at least 500 m (and often greater distances) away from each other. Salvaging entailed removal of all merchantable and damaged trees except for standing live *P. resinosa*, *P. strobus*, and *T. occidentalis* (USDA Forest Service 2000).

Semiochemical Treatments: Baited Pitfall Traps Rhizophagous insects and their associates were caught with baited pitfall traps that consisted of a 500 ml round plastic container (10.7 cm i.d.) inside a 1.0 l plastic liner buried in the ground (Spence and Niemelä 1994). Propylene glycol (200 ml; recreational vehicle antifreeze, Peak Co., Northbrook, IL, USA) in the plastic container served as killing agent and preservative for trapped beetles (Weeks and McIntyre 1997). The trap was covered with a 100 cm^2 plywood board suspended 4 cm above the trap to minimize flooding and disturbance by animals. Baits were hung from the plywood board and placed inside the inner cup above the level of propylene glycol. Baits included 4 host attractant solutions: 2% (−)-α-pinene, 2% (+)-α-pinene, 2% (−)-β-pinene (in ethanol), and a 100% ethanol control. The pinenes were purchased from Sigma Aldrich (St. Louis, MO, USA), and all had chemical purities of ≥99% and enantiomeric purities of >98%. The ethanol was

purchased from Aaper Alcohol Co. (Shelbyville, KY, USA).

During the summer of 2000, a total of 144 baited pitfall traps were installed in the ABC and JP cover types. There were 12 baited pitfall traps per site with three replicates of each bait treatment placed on a 6 by 2 grid, and separated by >20 m to reduce trap sampling interactions. The 12 traps were each baited with one of the four semiochemical blends. Semiochemical blends were released from wicks inserted in full 15 ml glass vials. When traps were emptied, the location of the trap and its semiochemical bait were not re-randomized within the site, and the baits were not replaced during the summer. In 2000, traps were operated from August to October and emptied every 15–30 d. For logistical reasons, the study was not initiated until August; for biological reasons, May would have been preferable.

During the summers of 2001–2003, the sampling design was changed to include 80 traps distributed over 16 sites, all located in the JP cover type. At each site, 5 pitfall traps baited with the 4 semiochemical blends in ethanol, ethanol alone, and an unbaited control were assigned to 5 sample sub-plots randomly drawn from a grid of 28 sub-plots. When the traps were emptied, the inner cups and the associated baits were re-randomized on the 5 outer cup locations (i.e., among the 5 sub-plots). In 2001 and 2002, the semiochemical blends were released from closed polyethylene bottles at an unknown low release rate (15 ml total volume) [Phero Tech. (now Contech Enterprises) Inc., Delta, BC, Canada]. In 2003, semiochemical blends were released from these polyethylene bottles with a 0.13 cm^2 hole in the side of the vial cap. The mean (± SE) release rates for 15 d in the field for each of the semiochemical blends in 2003 were as follows: (1) (−)-α-pinene: 7.98±0.23 g; (2) (+)-α-pinene: 7.64±0.26 g; (3) (−)-β-pinene: 7.24±0.20 g; and (4) 100% ethanol: 7.48± 0.20 g. Baiting started in May, and baits were replaced in July in 2001 and 2002, and every 2 wk in 2003. In 2001–2003, traps were deployed from May to October and emptied every 15–20 d.

Semiochemical Treatments: Lindgren Funnel Traps Subcortical insects were caught in flight with 16-unit Lindgren funnel traps (Lindgren 1983). The traps were baited on the 8th funnel from the top with semiochemicals that were chosen to elicit responses from the major species of scolytid and woodboring beetles known to be present in this subboreal forest (Dodge 1938; Bright 1976; University of Minnesota Insect Collection (UMIC), St. Paul, MN; K.J.K.G., personal observation) (Table 2). The selection of many of the semiochemicals was based on research data accumulated on populations of scolytid and woodboring beetles from western North America (Table 2). A bait for the spruce beetle, *Dendroctonus rufipennis* (Kirby) was employed initially in 2000 and 2001 in anticipation of potential population increases of this species, but because we trapped far more individuals of the eastern larch beetle, *Dendroctonus simplex* LeConte than *D. rufipennis* in response to this bait, we used a different bait targeting *D. simplex* in 2002 and 2003 (see Discussion for further explanation). During the summer of 2000, a total of 324 funnel traps were installed in the ABC and JP forest cover types. Twenty seven funnel traps were hung on 2.2 m tall iron rebar poles in each site so that the trap collection cup was approximately 0.1 m above the forest floor. The traps were placed on a 7 by 4 grid, separated by >20 m, and were >20 m from forest edges. Traps were each baited with 1 of 8 semiochemical blends, or left unbaited (3 replicates of each semiochemical treatment and control at each site). The collection cup of each trap contained a 4 cm^2 Dicholorvos-impregnated plastic strip to kill insects. When traps were emptied, the location of the trap and its semiochemical bait were not re-randomized within the site. In 2000, traps were deployed from August to October and emptied every 15–30 d.

During the summer of 2001, the sampling design was altered to include 160 traps distributed over 16 sites located only in the JP cover type. At each site, 10 funnel traps (baited with 9 semiochemical combinations and an unbaited control) were assigned to 10 sample sub-plots randomly drawn from a grid of 28 sub-plots. During the summers of 2002–2003, the sampling design was altered further to include 176 traps distributed over 16 sites in the JP cover type. At each site, 11 funnel traps (baited with 10 semiochemicals and an unbaited control) were assigned to 11 sample sub-plots randomly drawn from a grid of 28 sub-plots. Semiochemical treatments were altered among years 2000, 2001, and 2002–2003 after assessing the trapping efficacy of the baits. Semiochemical baits were replaced each year in July or August. In 2001–2003, when traps were emptied every 15–20 d, the location of the trap and its semiochemical bait were re-randomized among the 10 or 11 sub-plots. In 2001–2003, traps were deployed from May to October and emptied every 15–20 d.

Adult subcortical insects were identified by K.J.K.G. with taxonomic keys (Middlekauff 1960; Linsley 1962a, b, 1963, 1964; Linsley and Chemsak 1972; Nelson 1975; Bright 1976, 1987; Wood 1982b, Triplehorn 1990; Goulet 1992; Chemsak 1996; Downie and Arnett 1996; Yanega 1996; Kovarick and Caterino 2001; Bellamy and Nelson 2002; Optiz 2002; Pollack 2002; Thomas 2002; Turnbow and Thomas 2002) and with the assistance of systematists. Specifically, reference collections of buprestid, cerambycid, clerid, histerid, scolytid, and tenebrionid beetles and siricid wasps were verified by C. Bellamy (California Department of Food and Agriculture, Sacramento) and G. Nelson (College of Osteopathic Medicine, Kansas City, deceased);

Table 2 List of semiochemicals used to target subcortical beetle species in Lindgren funnel traps in 2000–2003, Superior National Forest, Cook Co., MN, USA[a]

Target Beetle Species or Families	Semiochemical	Enantiomeric Composition	Load (mg)	Release rate/d	Chemical Purity (%)
Ips grandicollis[b]	Ipsenol[c]	97%-(−)	20	240 µg[d]	97
	α-Pinene[e]	98.5%-(−)	6,413	200–300 mg[d]	99
Ips perroti[b]	Ipsenol[c]	97%-(−)	20	240 µg[d]	97
	Ipsdienol[c]	97%-(−)	20	110 µg[d]	93
Ips perroti[f]	Ipsenol[c]	97%-(−)	20	240 µg[d]	97
	Ipsdienol[c]	97%-(+)	20	110 µg[d]	93
Ips perturbatus[b]	Ipsenol[c]	97%-(−)	20	240 µg[d]	97
	Ipsdienol[c]	97%-(+)	20	110 µg[d]	93
	cis-Verbenol[c]	83%-(−)	20	300–600 µg[d]	94
Ips pini[b]	Ipsdienol[c]	Racemic	20	110 µg[d]	93
	Lanierone[c]	NA	4	10 µg[d]	≥98
Dendroctonus rufipennis[g]	Frontalin[h]	Racemic	300	2.6 mg[i]	≥98
	α-Pinene[j]	96%-(−)	650	1.5 mg[k]	99
Dendroctonus simplex[g]	1-Methyl-2-cyclohexenol[c]	Racemic	20	1.5 mg[d]	97
	Seudenol[c]	Racemic	20	1.5 mg[l]	99
	α-Pinene[j]	96%-(−)	650	1.5 mg[k]	99
Dendroctonus valens[b]	α-Pinene[e]	98.5%-(+)	12,870	200–300 mg[d]	99
	β-Pinene[e]	(−)	12,990	200–300 mg[d]	99
Dendroctonus valens[m] Phero Tech Bait	α-Pinene[e]	(+)	4,290	unknown	
	β-Pinene[e]	(−)	4,330	unknown	
	3-Carene[e]	(+)	4,285	unknown	
Dryocoetes spp.[n]	*exo*-Brevicomin[o]	Racemic	243	1.7 mg[i]	97
	α-Pinene[e]	98.5%-(−)	6,435	200–300 mg[d]	99
Buprestidae, Cerambycidae[b]	Ethanol[p]	NA	120,000	400 mg[l]	≥98
	α-Pinene[p]	96%-(−)	170,000	2 g[l]	99

[a] All materials and release rates were provided by Phero Tech (now Contech Enterprises) Inc. (Delta, BC, Canada), except for (+)-α-pinene and (−)-β-pinene used for *D. valens*, which were purchased from Sigma Aldrich and formulated in our laboratory. References for the selection of semiochemical attractants are as follows: *Ips grandicollis* (Erbilgin and Raffa 2000); *Ips perroti* (Ayres et al. 2001); *Ips perturbatus* (Holsten et al. 2000); *Ips pini* (Teale et al. 1991); *Dendroctonus rufipennis* (Borden et al. 1996); *Dendroctonus simplex* (Werner et al. 1981); *Dendroctonus valens* (Hobson et al. 1993); and *Dryocoetes* spp. (Camacho et al. 1998)

[b] Baits were used in 2000–2003

[c] Bubble cap release device

[d] At 25°C

[e] 15 ml Polyethylene screw-cap bottle release device

[f] Bait was used in 2001–2003

[g] Bait for *D. rufipennis* was used in 2000 and 2001; bait for *D. simplex* was used in 2002 and 2003

[h] 400 µl Polyethylene Eppendorf vial release device

[i] At 23°C

[j] 1.8 mL Polyethylene Eppendorf vial release device

[k] At 21°C

[l] At 20°C

[m] Bait was used in 2002–2003

[n] *Dryocoetes* spp. were sampled with (+/−)-*endo*-brevicomin alone in 2000

[o] 250 µl Polyethylene Eppendorf vial release device

[p] Ultra high release device

NA- Not Applicable

J. Chemsak (University of California, Berkeley, deceased) and D. Penrose (California Department of Food and Agriculture, Sacramento); W. Barr (University of Idaho, retired) and J. Rifkind (private collector, Valley Village, California); Y. Bousquet (Agriculture Canada, Ottawa); D. Bright (Agriculture Canada, Ottawa, retired); C. Triplehorn (Ohio State University, retired); and J. Luhman (Minnesota Department of Food and Agriculture, St. Paul), respectively. All curculionid beetles were identified by S.J.K (University of Wisconsin) and D. Langor (Canadian Forest Service, Edmonton) (Blatchley and Leng 1916; Kissinger 1964; Fridrich 1965; Warner 1966; Salsbury 2000). Voucher specimens have been deposited at the UMIC, St. Paul, MN and the California Academy of Sciences, San Francisco, CA. The UMIC was also examined during this study for specimens of potential new state record insects that had not been recorded previously in the literature.

Data Handling and Statistical Analyses

Pitfall Traps Since only 500 beetles were caught in the pitfall traps during the summers of 2000–2003, no statistical analyses were performed. Instead, species-lists with a brief interpretation of trends are provided. Such small catches may represent either the inadequacy of the traps or baits or small population sizes in the forest stands.

Funnel Traps: Subcortical Insect Trap Catches Trap catches of early successional subcortical insects (=subcortical insects) were standardized to 15-d intervals [(trap catch/ total number of days that trap was operational) X 15] as the traps were emptied approximately every 2 wk. This standardization accounted for the variable number of days when the traps were operational in the plots. If funnel traps were disturbed for a particular period, catches from that period were excluded from analyses. For data collected in 2000, trap catches were pooled for all subcortical insects over the season, and three-factor analyses of variance (ANOVA) were performed first with the cover type, land-area treatment, and semiochemical treatment as factors (SAS 1999–2001). Data were checked for the required assumptions of constant variance and normality using residual and normality plots. A Goodness-of-Fit test for normal distribution (Kolmogorov-Smirnov) was conducted on the residuals to test the null hypothesis that the data were normally distributed. Data for standardized subcortical insect catches showed non-normality, and were transformed by ln (x+1), which resulted in normally distributed data. Type III Sum of Squares were used to assess the statistical significance of factors at α=0.05. The ANOVA was performed in an iterative manner in which the complete model was analyzed by successive deletions of non-significant higher-order interaction terms, two-way interaction terms, and main factors. The final model terms that were significant are presented in the tables, and all terms that were omitted as a consequence of the iterative procedure are listed as non-significant (NS). The Ryan-Einot-Gabriel-Welsch (REGW) test with α=0.05 was used for pairwise comparisons among treatment means (Day and Quinn 1989). All data were back-transformed before presentation.

For data collected in 2001–2003, split-plot analyses of variance tests were performed for: 1) all subcortical insects species combined; 2) subcortical insect families with ≥10% of total catch; and 3) subcortical insect species with ≥1% of the total beetle catch by using the PROC MIXED option (SAS 1999–2001). Split-plot analyses were performed because the traps were re-randomized every 15 d; the whole plot factor was the land-area treatment and the two split-plot factors were semiochemical treatment and year. The split-plot analysis of variance test for all subcortical insect species combined was also performed as described above, but excluding the trap catch of the most abundant subcortical insect species. This test was performed to assess the effect of the most abundant species on the total trap catches.

To determine if the responses of males and females of the four most abundant scolytid species were dependent upon the land-area treatment, selected semiochemical treatments, and year (2001–2003), a three- or a two-way ANOVA was performed on the standardized percentage of males trapped in funnel traps. These four species also were relatively easy to separate by sex. To assemble this data set, we determined the sex of 1,937 adults of *D. simplex*, 2,684 *I. grandicollis*, 7,442 *I. perroti*, and 8,811 *I. pini*. Trap catches with <10 individuals were excluded from these analyses because of the bias in sex ratio from small samples.

Funnel Traps: Subcortical Insect Species Richness Venn diagrams were used to illustrate relationships of species richness (total number of early successional species) among the ABC and JP cover types, and the land-area treatments. In the diagrams, each habitat set is represented by a circle that contains the unique numbers of species; overlapping areas depict numbers of insect species held in common with other cover type and land-area treatment combinations (Langbehn et al. 1972). Venn diagrams were constructed for species richness as follows: 1) 2000 funnel trap catches in the ABC (Fig. 4a) and JP (Fig. 4b) cover types in the three land-area treatments; and 2) 2001–2003 funnel trap catches (pooled over all three seasons) in the JP cover type in the four land-area treatments.

Funnel Traps: Subcortical Insect Species Diversity Two methods (rarefaction and Simpson's diversity index) were used to assess subcortical insect species diversity in funnel trap catches in relationship to the cover type, land-area

treatment, and semiochemical treatment. Rarefaction indices estimate the mean (± SE) subcortical insect species diversity (richness and evenness); the technique accounts for differences in trapping effort among habitats and is ideal for catches with variable trapping intervals (Magurran 2004; software developed by Holland 2003). Rarefaction analyses were performed on the following subcortical insect assemblages: 1) 2000 funnel trap catches in the ABC and JP cover types; 2) 2001–2003 funnel trap catches in 4 land-area treatments in the JP cover type; 3) 2000 funnel trap catches with 9 semiochemical treatments (including unbaited control) in the ABC and JP cover types; and 4) 2001–2003 funnel trap catches with 12 (including unbaited control) semiochemical treatments in the JP cover type.

Simpson's diversity index was used to assess species diversity and dominance for land-area and semiochemical treatments (Magurran 2004). The measure was expressed as "diversity" rather than "dominance" to ensure that species diversity increased and dominance decreased with an increasing index value (McCune and Mefford 1999; McCune and Grace 2002).

Funnel Traps: Subcortical Insect Species Composition Subcortical insect species compositions for each land-area treatment, year, and semiochemical treatment for JP stands in 2001–2003 were compared by constructing dendrograms with a similarity percentage scale that was generated by cluster analysis. In this clustering technique, standardized mean funnel trap catches within each treatment-type were analyzed by using the Bray-Curtis Distance method with the group average option (McCune and Mefford 1999; McCune and Grace 2002).

Results

Pitfall Trap Sampling During the summers of 2000–2003, 1,828 pitfall trap samples yielded 500 rhizophagous beetle adults representing 5 families, 34 genera, and 44 species (Supplemental Data Table 1). In 2000, only one species [*Dryocoetes autographus* (Ratzeburg) (Scolytidae)] was trapped in the ABC cover type. In the JP cover type, Curculionidae (73% of the total catch) was the most abundant family followed by Scolytidae (19%), and Cerambycidae (7%). Within each of these respective families, *Hylobius pales* (Herbst) (27%), *Hylurgops rugipennis pinifex* (Fitch) (8%), and *Stictoleptura canadensis canadensis* (Olivier) (2%) were the most abundant. Overall, *H. pales* (27%), *Hylobius radicis* Buchanan (16%), and *Otiorhynchus ovatus* (Linnaeus) (9%) (all Curculionidae) were the most abundant beetle species. Thirty-two rhizophagous beetle species were rare and represented ≤1% of the total beetle catch; ten rhizophagous beetle species were common and represented 1–10% of total beetle catch; and two rhizophagous beetle species were abundant and represented >10% of the total beetle catch. *Otiorhynchus ovatus* and *Polydrusus sericeus* (Schaller) are exotic species from Europe. Several species of bark beetles [e.g., *Ips grandicollis* (Eichhoff), *Ips pini* (Say), *Dr. autographus*, *Polygraphus rufipennis* (Kirby), and *Dendroctonus simplex* LeConte] generally not associated with the forest floor were caught in these traps.

In 2000–2003, 42% of the beetles were caught in the burned sites followed by lower percentages in the undisturbed (21%), salvaged (21%), and wind-disturbed (16%) sites. The species richness was also highest in the burned sites (26 species). Most of the rhizophagous beetles were caught in the traps baited with ethanol solutions of (+)-α-pinene (33%) followed by traps baited with (−)-α-pinene (26%), (−)-β-pinene (23%), ethanol (11%), and unbaited traps (7%) (Supplemental Data Table 2). The species richness (23 species) was also highest in the traps baited with ethanol solutions of (+)-α-pinene.

Funnel Trap Sampling During the summers of 2000–2003, 3,975 funnel trap samples yielded a total of 86,471 subcortical insect adults representing 12 families, 96 genera, and 143 species (Supplemental Data Tables 3, 4). The majority of subcortical insect species (126) were rare (i.e., each represented ≤1% of the total insect catch), 14 subcortical insect species were common (i.e., each represented 1–10% of the total insect catch), and three subcortical insect species were abundant (i.e., each represented >10% of the total insect catch).

Scolytidae (55% of the total catch) was the most abundant family followed by Cleridae (18%), Buprestidae (13%), and Cerambycidae (10%). Other families such as Anthribidae, Cucujidae, Curculionidae, Histeridae, Nemonchyidae, Salpingidae, Siricidae, and Tenebrionidae each represented <5% of the total catch. Within their respective families, *D. simplex* (35%) (Scolytidae), *Thanasimus dubius* (Fabricius) (65%) (Cleridae), *Buprestis maculativentris* Say (10%) (Buprestidae), and *Monochamus scutellatus scutellatus* (Say) (25%) (Cerambycidae) were the most abundant species. Overall, *D. simplex* (19%) was the most abundant beetle species followed by *T. dubius* (12%), and *I. pini* (10%).

Among families, cerambycid beetles had the greatest species diversity (43 species) followed by curculionid (34 species), scolytid (24 species), and buprestid (21 species) beetles. We also caught 10 species of clerid beetles and 5 species of siricid woodwasps. Among buprestid beetles, *Chrysobothris sexsignata* (Say), *Cypriacis striata* (Fabricius), *Cypriacis sulcicollis* (LeConte), *Phaenops aeneola* (Melsheimer), and *Phaenops drummondi drummondi* (Kirby) are new state records (Downie and Arnett 1996).

Among cerambycid beetles, *Pygoleptura nigrella nigrella* (Say) and *Xestoleptura tibialis* (LeConte) are new state records (Linsley 1962a, b, 1963, 1964; Linsley and Chemsak 1972; Downie and Arnett 1996; Yanega 1996). Among clerid beetles, *Enoclerus muttkowski* (Wolcott), *Enoclerus nigripes rufiventris* (Spinola), *Madoniella dislocatus* (Say), *Thanasimus trifasciatus* (Say), *Thanasimus nubilus* (Klug), *Thanasimus undatulus undatulus* (Say), *Trichodes nutalli* (Kirby), and *Zenodosus sanguineus* (Say) are new state records (Downie and Arnett 1996). Katovich and Ostry (1998) reported that *Z. sanguineus* was collected on the bark of butternut, *Juglans cinerea*, but it was not clear whether the species was collected from Wisconsin or Minnesota. Among histerid beetles, *Paromalus teres* LeConte, *Platysoma coarctatum* LeConte, *Platysoma deficiens* Casey, and *Platysoma lecontei* (Marseul) are new state records (Downie and Arnett 1996). Among salpingid beetles, *Boros unicolor* Say is a new state record (Downie and Arnett 1996), whereas among scolytid beetles, *Ips latidens* (LeConte) is a new regional record for the Great Lakes region in the USA (Dodge 1938; Wood 1982b; Downie and Arnett 1996). Among siricid wasps, *Sirex behrensii* (Cresson), *Urocerus gigas flavicornis* (Fabricius), and *Xeris spectrum* (Linnaeus) are new state records (Smith and Schiff 2002). Among these new records, *C. sulcicollis*, *P. aeneola, E. muttkowski*, *E. n. rufiventris, T. trifasciatus, I. latidens,* and *S. behrensii* were not present in the University of Minnesota Insect Collection, whereas the rest of the species had been collected previously from Minnesota and deposited in the museum, but not reported in the literature (Supplemental Data Table 5).

Subcortical Insect Trap Catches The three-way ANOVA for pooled trap catches of all subcortical insect species for the year 2000 showed that cover type, land-area treatment, and semiochemical treatment were significant main effects in the model (Table 3, Fig. 1). Thirty five percent more subcortical insects were caught in the JP than in the ABC cover type (Fig. 1a). Across both cover types significantly more (20–34%) insects were caught in the salvaged than in the undisturbed and wind-disturbed forests (Fig. 1a). Significantly more subcortical insects of all taxa were caught in response to the *I. pini* bait than to the other baits (Fig. 1b), and significantly more (478–559%) insects were caught in response to the *Dendroctonus rufipennis*, *I. grandicollis*, and *I. perturbatus* (Eichhoff) baits than to the unbaited traps (Fig. 1b).

For all subcortical insect species for 2001–2003, there were significant interactions between year and land-area treatment, and between year and semiochemical treatment (Table 3, Supplemental Data Fig. 1). In general, trap catches were up to 2.5 times greater in 2001 and 2002 than in 2003, except in the undisturbed forests. In 2002, the *D. simplex* bait caught more beetles than the rest of the bait-types. In 2001–2003, fifteen subcortical beetle species in order of decreasing catches from funnel traps included *D. simplex*, *T. dubius*, *I. pini*, *I. perroti*, *I. perturbatus*, *B. maculativentris*, *I. grandicollis*, *T. u. undatulus*, *T. nubilus*, *Monochamus s. scutellatus*, *M. mutator* LeConte, *Dr. autographus*, *P. rufipennis*, *Chalcophora virginiensis* (Drury), and *H. r. pinifex* (Table 3). Significant interactions between and among model factors occurred, but varied among the families (Table 3, Supplemental Data Figs. 2, 3 and 4) and species (Table 3, Supplemental Data Figs. 5, 6, 7, 8, 9, 10, 11, 12 and 13). *Ips grandicollis* and *I. perturbatus* had significant three-way interactions among model factors (Table 3), which could not be displayed graphically. In 2001 in burned forests, significantly more *B. maculativentris*, *T. nubilus*, and *P. rufipennis* were caught than in 2002 and 2003; in 2001 and 2002 in salvaged forests, significantly more *C. virginiensis* were caught than in 2003; and in 2001 in wind-disturbed forests, more *M. s. scutellatus*, *I. pini*, and *T. dubius* were caught than in 2002 and 2003. In general, significantly more *D. simplex* (Fig. 2a), *I. pini* (Fig. 2b), *I. perroti* (Fig. 2c), *I. perturbatus* (Fig. 2d), and *I. grandicollis* (Fig. 2e) were trapped in response to their respective baits than to the other semiochemical combinations, although the response of *I. grandicollis* tended to be more generic to the baits that we tested. The predaceous beetle, *T. dubius*, also tended to respond more generally to a variety of the baits that we tested (Fig. 2f), perhaps underscoring its role as a generalist bark beetle predator. *Ips perroti* responded more to the *I. perroti*-(−)-ipsdienol bait (Fig. 2c); *H. r. pinifex* (Fig. 2g) to the *D. valens*-Phero Tech and woodborer baits; *T. dubius* to the *D. rufipennis* bait (Fig. 2f); and *Dr. autographus* (Fig. 2h) to the *Dryocoetes* spp. and woodborer baits.

The grand mean percentage of male *D. simplex* trapped in 2001–2003 was 49%. Year was not used as a model factor in the analysis because of low trap catches in years 2001 and 2003. Because of the higher response of *D. simplex* (Fig. 2a), the trap catches analyzed included those responding to the baits targeting *D. simplex*, *I. pini*, and woodborers. The two-way ANOVA for the percentage of male *D. simplex* indicated no significant effect of the interactions of land-area and semiochemical treatments ($F_{(4,35)}$ = 1.89, P=0.141), or of land-area treatment alone ($F_{(2,35)}$ = 0.48, P=0.625) or semiochemical treatment alone ($F_{(2,35)}$ = 3.14, P=0.056).

The grand mean percentage of male *I. pini* trapped in 2001–2003 was 44%. Because of the higher response of *I. pini* (Fig. 2b), the trap catches analyzed included those responding to the baits targeting *I. pini* and *I. perroti*-(+)-ipsdienol. The three-way ANOVA for the percentage of male *I. pini* indicated no significant effect of any of the second or third-order interactions (P>0.5), of year alone

Table 3 ANOVA model for the pooled funnel trap catches of all subcortical beetle species, four beetle families, and fourteen species with year (YR), land-area treatment (LT), semiochemical treatment (ST), and their interactions as factors, Superior National Forest, Cook Co., MN, USA

Source[a]	YR	LT	ST	YR*LT	YR*ST	LT*ST	YR*LT*ST
All Subcortical Insects 2000[b]	NA	$10.44_{2,107}$ <0.001	$9.99_{8,107}$ <0.001	NA	NA	NS	NA
All Subcortical Insects 2001–2003[c]	$24.84_{2,3573}$ <0.001	$9.69_{3,3573}$ <0.001	$8.87_{11,3573}$ <0.001	$4.36_{6,3573}$ <0.001	$8.49_{22,3573}$ <0.001	NS	NS
Buprestidae[d]	$179.55_{2,3573}$ <0.001	$124.11_{3,3573}$ <0.001	NS	$64.48_{6,3573}$ <0.001	NS	NS	NS
Cerambycidae[d]	$75.9_{2,3573}$ <0.001	$25.86_{3,3573}$ <0.001	$43.49_{11,3573}$ <0.001	$13.4_{6,3573}$ <0.001	$5.25_{22,3573}$ <0.001	NS	$1.27_{66,3573}$ 0.048
Cleridae[d]	$4.38_{2,3573}$ 0.013	$49.83_{3,3573}$ <0.001	$87.12_{11,3573}$ <0.001	$7.8_{6,3573}$ <0.001	$4.36_{22,3573}$ <0.001	$10.65_{33,3573}$ <0.001	NS
Scolytidae[d]	$16.95_{2,3573}$ <0.001	$4.4_{3,3573}$ 0.004	$8.73_{11,3573}$ <0.001	$2.26_{6,3573}$ 0.035	$4.23_{22,3573}$ 0.002	$1.89_{33,3573}$ 0.002	NS
Buprestis maculativentris[d]	$49.73_{2,3573}$ <0.001	$96.48_{3,3573}$ <0.001	NS	$25.44_{6,3573}$ <0.001	NS	NS	NS
Chalcophora virginiensis[d]	$44.98_{2,3573}$ <0.001	$96.19_{3,3573}$ <0.001	NS	$25.4_{6,3573}$ <0.001	NS	NS	NS
Dendroctonus simplex[d]	$15.82_{2,3573}$ <0.001	NS	$9.4_{11,3573}$ <0.001	NS	$5.05_{22,3573}$ <0.001	$1.53_{33,3573}$ 0.027	NS
Dryocoetes autographus[d]	NS	$6.07_{3,3573}$ <0.001	$27.01_{11,3573}$ <0.001	$2.51_{6,3573}$ 0.02	$3.11_{22,3573}$ <0.001	$3.69_{33,3573}$ <0.001	NS
Ips grandicollis[d]	$20.45_{2,3573}$ <0.001	$5.1_{3,3573}$ 0.002	$62.6_{11,3573}$ <0.001	$5.84_{6,3573}$ <0.001	$4.08_{22,3573}$ <0.001	NS	$1.55_{66,3573}$ 0.001
Ips perroti[d]	$3.45_{2,3573}$ 0.032	$5.16_{3,3573}$ 0.002	$52.79_{11,3573}$ <0.001	NS	$2.23_{22,3573}$ 0.002	$5.38_{33,3573}$ <0.001	NS
Ips perturbatus[d]	$6_{2,3573}$ 0.003	$9.02_{3,3573}$ <0.001	$18.99_{11,3573}$ <0.001	$3.38_{6,3573}$ <0.001	$4.09_{22,3573}$ <0.001	$5.45_{33,3573}$ <0.001	$2.61_{66,3573}$ <0.001
Ips pini[d]	$4.24_{2,3573}$ 0.015	$2.77_{3,3573}$ 0.04	$34.54_{11,3573}$ <0.001	$2.28_{6,3573}$ 0.034	NS	$2.49_{33,3573}$ <0.001	NS
Monochamus mutator[d]	$29.91_{2,3573}$ <0.001	$10.45_{3,3573}$ <0.001	$30.22_{11,3573}$ <0.001	$4.8_{6,3573}$ <0.001	$3.29_{22,3573}$ <0.001	$3.73_{33,3573}$ <0.001	$1.77_{66,3573}$ <0.001
Monochamus s. scutellatus[d]	$33.64_{2,3573}$ <0.001	$33.5_{3,3573}$ <0.001	$7.77_{11,3573}$ <0.001	$9.06_{6,3573}$ <0.001	$1.76_{22,3573}$ 0.03	NS	NS
Polygraphus rufipennis[d]	$15.77_{2,3573}$ <0.001	$11.2_{3,3573}$ <0.001	NS	$10.65_{6,3573}$ <0.001	NS	NS	NS
Thanasimus dubius[d]	$19.23_{2,3573}$ <0.001	$47.52_{3,3573}$ <0.001	$102.74_{11,3573}$ <0.001	$2.6_{6,3573}$ 0.016	$4.1_{22,3573}$ <0.001	$12.25_{33,3573}$ <0.001	NS
Thanasimus u. undatulus[d]	$6.33_{2,3573}$ 0.002	$60.51_{3,3573}$ <0.001	$32.59_{11,3573}$ <0.001	$2.8_{6,3573}$ 0.01	$2.6_{22,3573}$ <0.001	$6_{33,3573}$ <0.001	NS
Thanasimus nubilus[d]	$81.53_{2,3573}$ <0.001	$47.95_{3,3573}$ <0.001	NS	$45.81_{6,3573}$ <0.001	NS	NS	NS

[a] For each table entry, the top number is the *F*-value, the bottom number is the *P*-value, and the subscripts refer to the degrees of freedom for each *F*-value. *NA* Non-applicable in the model; *NS* Non-significant factor in the model

[b] The ANOVA model was as follows: $Y_{ijk} = CT_i + LT_j + ST_k + (CT*LT)_{ij} + (CT*ST)_{ik} + (LT*ST)_{jk} + (CT*LT*ST)_{ijk} + \epsilon_{ijk}$ where Y_{ijk} is the observed trap catch for cover type, land-area treatment and semiochemical treatment; $_{i,\,j,\,k}$ are the numbers of levels for each factor; and ϵ_{ijk} is the error term (unaccounted variation in the model). Cover type was significant ($F=14.53_{2,107}$, $P=0.002$); none of the second- and third-order interactions with the cover type were significant

[c] The ANOVA model was as follows: $Y_{ijk} = LT_i + \eta_i + ST_j + (LT*ST)_{ij} + YR_k + (LT*YR)_{ik} + (ST*YR)_{jk} + (LT*ST*YR)_{ikj} + \epsilon_{ijk}$ where Y_{ijk} is the observed trap catch for land-area treatment, semiochemical treatment and year; $_{i,\,j,\,k}$ are the numbers of levels for each factor; η_i is the whole plot level random error term; and ϵ_{ijk} is the split plot level random error term

[d] Data from 2001–2003

($F_{(2,106)} = 0.46$, $P=0.634$), of land-area treatment alone ($F_{(3,106)} = 1.19$, $P=0.32$), or semiochemical treatment alone ($F_{(1,106)} = 0.25$, $P=0.615$).

The grand mean percentage of male *I. perroti* trapped in 2001–2003 was 25% (Fig. 3a). Because of the higher response of *I. perroti* (Fig. 2c), the trap catches analyzed included only those from traps baited with (−)-ipsdienol and (−)-ipsenol. The two-way ANOVA for the percentage of male *I. perroti* indicated significant effects of year ($F_{(2,103)} = 5.01$, $P=0.009$) and land-area treatment ($F_{(3,103)} =$

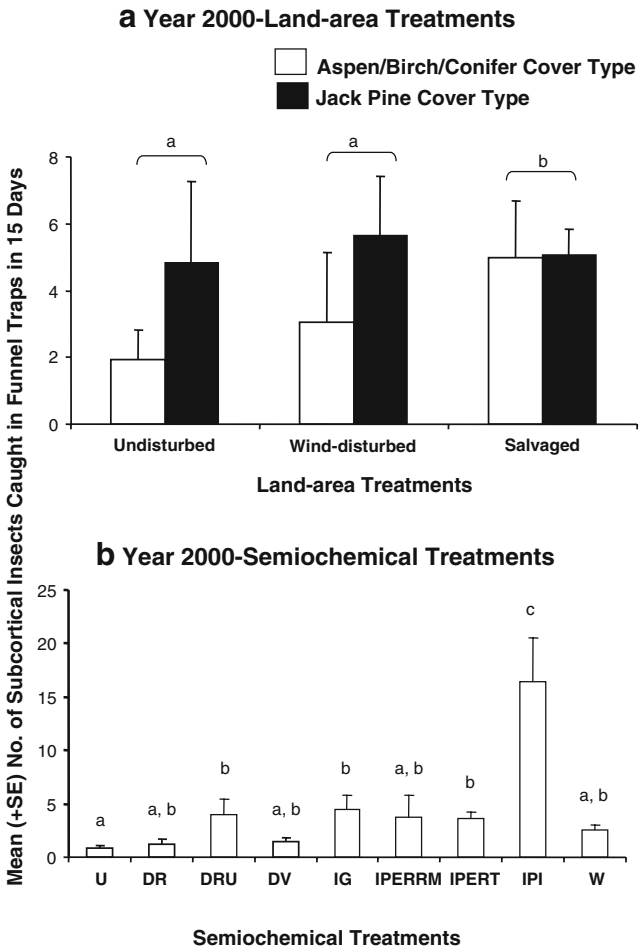


Fig. 1 Mean (+ SE) number of subcortical insects per 15 d caught during 2000 in funnel traps in the undisturbed, wind-disturbed, and salvaged plots in the aspen/birch/conifer and jack pine forest cover types (**a**). Mean (+ SE) number of subcortical insects per 15 d caught during 2000 in baited (eight semiochemical lures) or unbaited funnel traps in the aspen/birch/conifer and jack pine cover types (**b**). Key: U-Unbaited; DR-*Dryocoetes* spp.; DRU-*Dendroctonus rufipennis*; DV-*Dendroctonus valens*; IG-*Ips grandicollis*; IPERRM-*Ips perroti*; IPERT-*Ips perturbatus*; IPI-*Ips pini*; W-Woodborers (N=18). Different letters above histogram bars indicate significantly different means (Ryan-Einot-Gabriel-Welsch test, α=0.05). In (**a**) the comparisons were made among land-area treatments pooled across cover types

3.16, P=0.028) (Fig. 3a). Across most land-area treatments the percentage of males trapped was higher in 2001 than in 2002 and 2003 (38–50%). The percentage of males trapped was significantly higher in the burned sites than in the rest of the land-area treatments (Fig. 3a).

The grand mean percentage of male *I. grandicollis* trapped in 2001–2003 was 26% (Fig. 3b). Because of the higher response of *I. grandicollis* (Fig. 2e), the trap catches analyzed included only those responding to the bait targeting *I. grandicollis*. The two-way ANOVA for the percentage of male *I. grandicollis* indicated a significant effect of year ($F_{(2,49)}$ = 10.11, P<0.001) (Fig. 3b). There was no effect of land-area treatment ($F_{(3,49)}$ = 0.39, P=0.761) and no interaction between year and land-area treatment ($F_{(6,49)}$ = 0.83, P=0.554). The percentage of males trapped was significantly higher in 2003 than in 2001 and 2002 (by 50%).

Subcortical Insect Species Richness In 2000, 57 and 67 species of subcortical insects were caught in the ABC and JP cover types, respectively. A comparison of species richness among land-area treatments in the two cover types (Fig. 4) reveals that in 2000 the salvaged forests contained both the greatest total (49 and 51 species) and the most unique insect species (18 and 12 species) when compared with the undisturbed and wind-disturbed forests. In 2000, only 33% and 34% of the insect species were common to all land-area treatments in the ABC and JP cover types, respectively.

In 2001–2003, a total of 140 species of subcortical insects were caught in the JP cover type. A comparison of species richness among land-area treatments reveals that the burned (110 species) and wind-disturbed (106 species) plots contained both the greatest numbers and the most unique insect species (18 and 14 species) (Fig. 5). Fifty three percent to 58% of the subcortical insect species were shared among the four land-area treatments in the JP cover type. The greatest numbers of insect species were shared among the wind-disturbed, salvaged, and burned forests (81 species).

Subcortical Insect Species Diversity Rarefaction analyses of subcortical insect trap catches from 2000 by land-area treatment showed that at a sub-sample size of 400 individuals, the ABC and JP salvaged forests had the greatest estimated mean species diversities (Supplemental Data Fig. 14). Subcortical insect trap catches from 2001–2003 in the JP cover type by land-area treatment showed that at a sub-sample size of 14,000 individuals, the wind-disturbed and burned forests had the greatest estimated mean species diversity (Supplemental Data Fig. 15). Sub-sample sizes of 400 and 14,000 individuals were chosen for 2000 and 2001–2003, respectively, for comparisons across land-area treatments because they were the lowest sub-sample sizes at which a land-area treatment (ABC and JP undisturbed forest areas, respectively) stopped accumulating more species.

Simpson's diversity indices for subcortical insects in 2000 indicated that the salvaged forests of both cover types had the greatest species diversity (Table 4). Simpson's diversity indices for subcortical insects in 2001–2003 indicated that all land area treatments in the JP cover type had similar levels of subcortical insect species diversity, but numerically, the wind-disturbed forests had the greatest species diversity followed closely by the undisturbed, burned, and salvaged forests (Table 4).

Rarefaction analyses of subcortical insect trap catches from 2000 by semiochemical treatment showed that at a

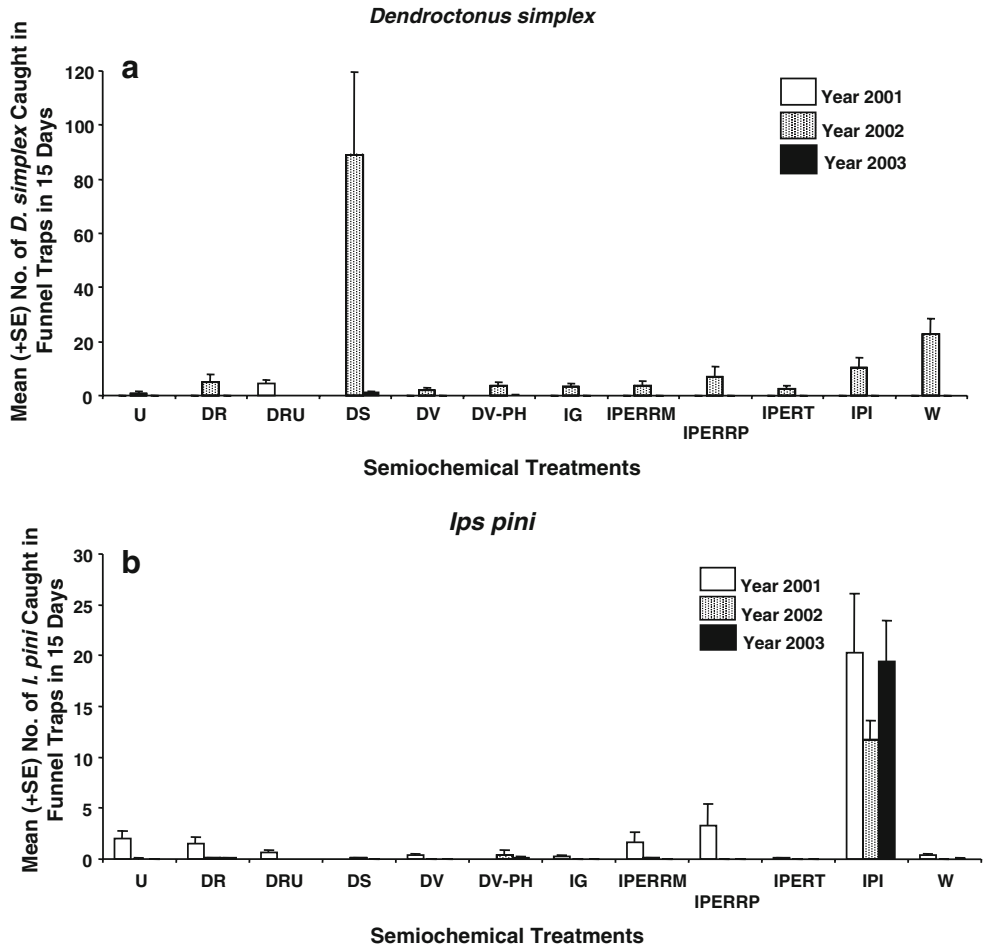


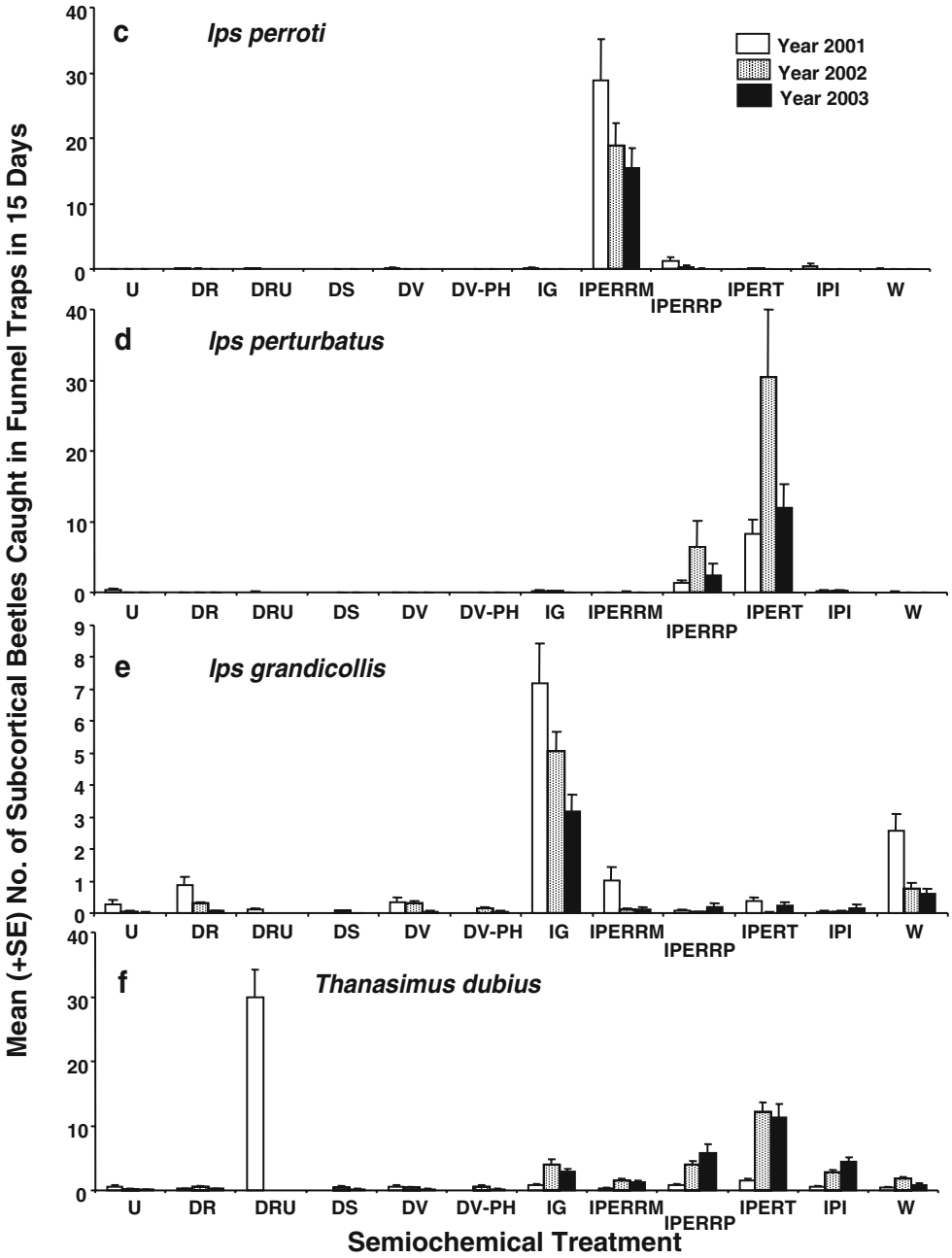
Fig. 2 Mean (+ SE) number of *Dendroctonus simplex* (**a**), *Ips pini* (**b**), *Ips perroti* (**c**), *Ips perturbatus* (**d**), *Ips grandicollis* (**e**), *Thanasimus dubius* (**f**), *Hylurgops rugipennis pinifex* (**g**), and *Dryocoetes autographus* (**h**) per 15 d caught during 2001–2003 in baited (11 semiochemical lures) or unbaited funnel traps in the jack pine cover type. Key: U- Unbaited control; DR- *Dryocoetes* spp.; DRU- *Dendroctonus rufipennis* (2001 only); DS- *Dendroctonus simplex* (2002 and 2003 only); DV- *Dendroctonus valens*; DV-PH- *Dendroctonus valens*-Phero Tech (2002 and 2003 only); IG- *Ips grandicollis*; IPERRM- *Ips perroti*-(−)-Ipsdienol; IPERRP- *Ips perroti*-(+)-Ipsdienol; IPERT- *Ips perturbatus*; IPI- *Ips pini*; W- Woodborers. For 2001, *N* ranges from 103 to 108; for 2002, *N* ranges from 100 to 109; and for 2003, *N* ranges from 86 to 93 for each semiochemical treatment. Apparent response shifts by *D. simplex* (**a**, 2001–2002); *T. dubius* (**f**, 2001–2002); and *H. r. pinifex* (**g**, 2001–2002) resulted as a consequence of the introduction to the experiment of the DS bait (2002); the removal of the DRU bait (2002); and the introduction of the DV-PH bait (2002), respectively

sub-sample size of 80 individuals, the *D. valens* bait elicited the numerically greatest estimated mean species diversity (Supplemental Data Fig. 16). Rarefaction analyses of subcortical insect trap catches from 2001–2003 by semiochemical treatment showed that at a sub-sample size of 1,500 individuals, the *Dryocoetes* spp. bait (Supplemental Data Fig. 17) elicited the numerically greatest estimated mean species diversity. Similar to the results from year 2000, the *I. pini* bait tended to accumulate more species with increasing sample sizes.

Simpson's diversity indices for subcortical insects in 2000–2003 indicated that the *Dryocoetes* spp. bait elicited the greatest species diversity followed by the *D. valens* and *D. valens*-Phero Tech baits, and woodborer baits, the unbaited trap, and various other baits (Table 5). The species diversities recorded from traps with the *I. pini* bait, the *I. perroti* (−)-ipsdienol bait, and the *D. simplex* bait were the lowest, likely reflecting the specificity of these baits.

Subcortical Insect Species Composition The cluster analysis of subcortical insect species composition among JP land-area treatment combinations in 2001–2003 revealed that there were two distinct groups: 1) the 2002 salvaged and burned forests with 90% similarity; and 2) the rest of the land-area treatments over the 3-year period of the study (Fig. 6). There was little similarity in species composition between the 2002 salvaged and burned forests and the rest of the groups. Within the second group, 2001 burned forests were 20%; 2003 salvaged and burned forests (with 85% similarity) and 2001 salvaged forests were 55%

Fig. 2 (continued)



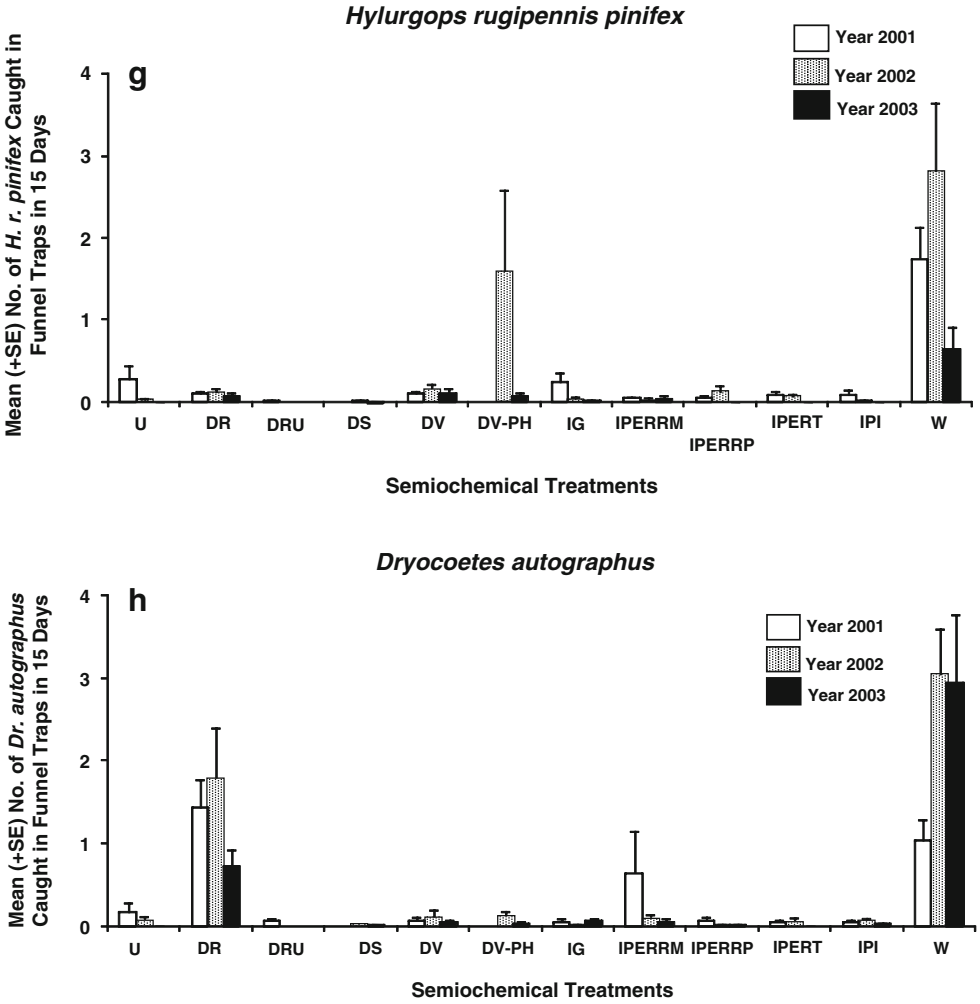
similar to the rest of the group. The undisturbed and wind-disturbed forests clustered together with >65% similarity in species composition. There was a 100% similarity between 2002 undisturbed and wind-disturbed forests.

Bray-Curtis cluster analyses of the compositions of subcortical beetle species responding to semiochemical baits in 2001–2003 revealed that the traps baited with attractants for *D. simplex* or woodborers had the most distinct species composition with only 2% similarity with the trap catches to the rest of the baits (Fig. 7). The species compositions responding to the *I. grandicollis* and *I. perroti*-(+)-ipsdienol baits were 85% similar, whereas those to the *D. valens* and *Dryocoetes* spp. baits and unbaited traps were 90% similar. There was almost 100% similarity between the trap catches to unbaited traps and the *D. valens*-baited traps.

Discussion

Our study of subcortical forest insect assemblages spanned 4 years with continuous summer trapping on a 120 km^2 stretch of sub-boreal forest landscape. We collected over 5,000 trap samples with 86,471 rhizophagous and subcortical insects represented by 143 species in baited Lindgren

Fig. 2 (continued)



funnel and pitfall traps. Other studies in North America on subcortical beetles of similar scope are by Hammond (1997) and Hammond et al. (2001) where 40,049 beetles represented by over 250 species were collected in 3 years from aspen, *Populus* spp., stands in north-central Alberta, Canada; and by Jacobs (2004) where 18,374 beetles represented by over 230 species were collected in 3 years from boreal deciduous and coniferous stands in northern Alberta. Neither of these studies used semiochemicals as baits to target key species in the trap catches. Although we caught a greater number of beetles in our traps, we had lower species richness than these other studies on subcortical insects. Possible reasons include: 1) inclusion of late-successional beetle taxa (e.g., Colydiidae, Elateridae, Passalidae, and Stapylinidae) by previous researchers in their analyses in contrast to inclusion of only early-successional taxa in our study (Savely 1939; Gibb et al. 2005); 2) the use of window-traps attached directly to the trees by previous researchers in contrast to baited Lindgren funnel traps hung from poles in our study (e.g., Sverdrup-Thygeson and Birkemoe (2008) found that window traps hung directly on aspen trees caught more beetles associated with aspen than traps hanging freely); and 3) some of the highly specific semiochemical baits that we used attracted large numbers of target species, but captured far fewer taxa than did unbaited traps or more generically baited traps.

The subcortical insect guild that we have characterized likely originated primarily from jack pine (*P. banksiana*) [e.g., *I. grandicollis*, *I. perroti, I. pini, Pityogenes plagiatus plagiatus* (LeConte) (all Scolytidae) and *M. s. scutellatus* (Cerambycidae)], with identifiable components from *L. laricina*, *Picea glauca*, *P. resinosa*, and *P. strobus*. For example, *D. simplex*, *Pityokteines sparsus* (LeConte), *P. rufipennis*, and *Scolytus piceae* (Swaine) (all Scolytidae), and *Phymatodes dimidiatus* (Kirby) and *Xylotrechus undulatus* (Say) (both Cerambycidae) have been reared or collected frequently from *L. laricina* (Blackman and Stage 1918; Dodge 1938; Linsley and Chemsak 1997). Similarly, *Crypturgus borealis* Swaine, *D. rufipennis*, *I. perturbatus*, *P. rufipennis*, and *Dryocoetes affaber* (Mannerheim) (all Scolytidae); and *Monochamus* spp. and *Tetropium cinnamopterum* Kirby (both Cerambycidae) have been reared or

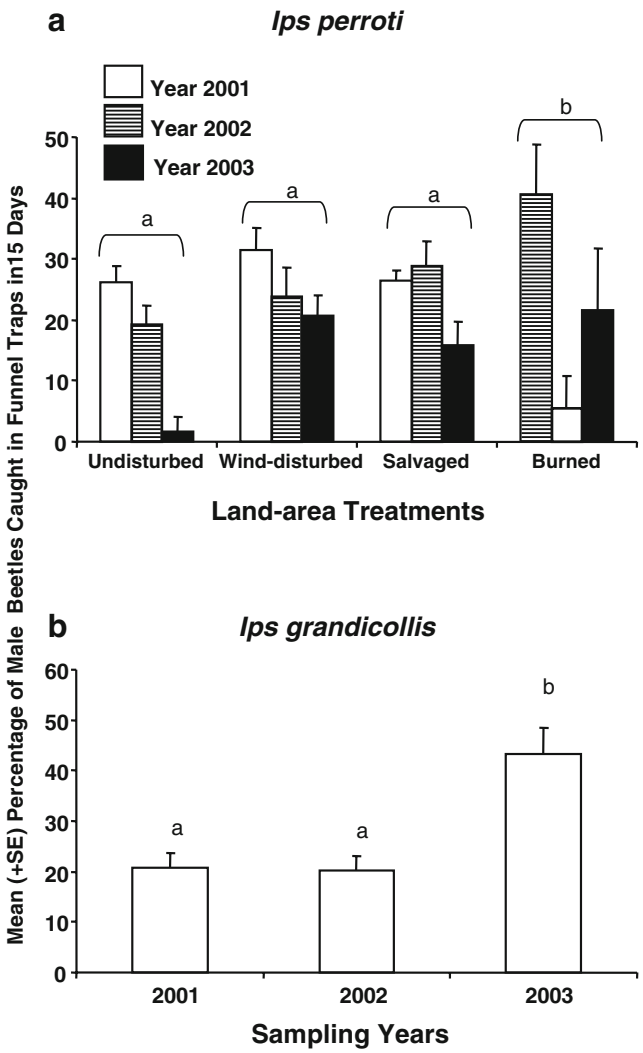


Fig. 3 **a** Percentage male (+ SE) *Ips perroti* per 15 d caught in funnel traps during 2001–2003 in the undisturbed, wind-disturbed, salvaged, and burned plots in the jack pine cover type (N=104). **b** Percentage male (+ SE) *Ips grandicollis* per 15 d caught in funnel traps during 2001–2003 in the jack pine cover type (N=50). Different letters above histogram bars indicate significantly different means (Ryan-Einot-Gabriel-Welsch test, α=0.05). In (**a**) the comparisons were made among land-area treatments pooled across years

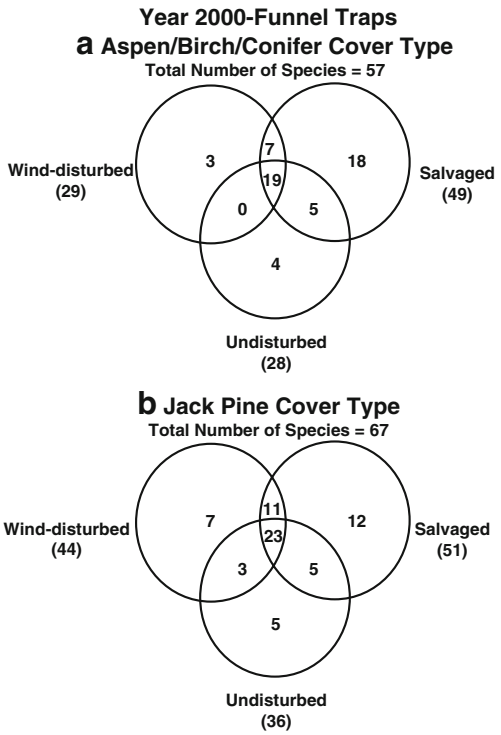


Fig. 4 Venn diagram depicting the total number of subcortical insect species trapped in funnel traps in 2000, and shared by or unique to the undisturbed, wind-disturbed, and salvaged plots in the aspen/birch/conifer **a** and jack pine **b** cover types. Values in parentheses below each land-area treatment refer to the total number of species collected in each habitat

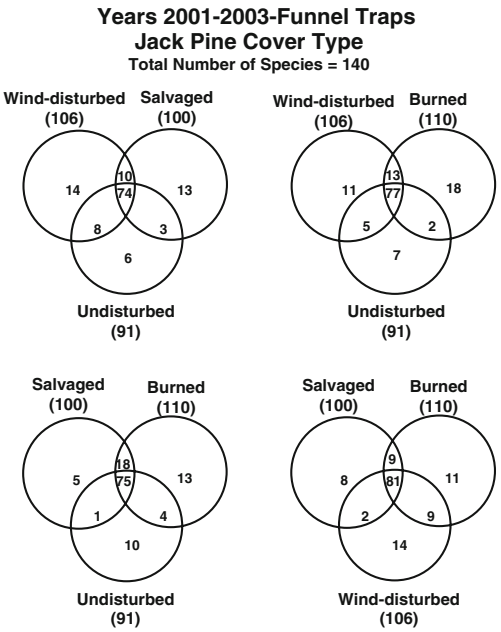


Fig. 5 Venn diagram depicting the total number of subcortical insect species trapped in funnel traps in 2001–2003, and shared by or unique to the undisturbed, wind-disturbed, salvaged, and burned plots in the jack pine cover type. Values in parentheses refer to the total number of species collected in each habitat

collected frequently from *P. glauca* (Dodge 1938; Whitmore 1982; Gara et al. 1995; Linsley and Chemsak 1997; Haberkern et al. 2002). *Ips latidens* and *I. pini* (Scolytidae) occur in *P. strobus* (Dodge 1938; Bright 1976; Wood 1982b; Drooz 1985). Other subcortical taxa such as *D. valens* and *Trypodendron lineatum* (Olivier) (both Scolytidae), as well as most Buprestidae, Cerambycidae, and Siricidae are extremely polyphagous; rearing studies from bark samples or cut logs are needed to establish host relationships.

Compared with subcortical insects caught in funnel traps, we caught few individuals and species of rhizophagous beetles in pitfall traps in 2000–2003. Such a result is

Table 4 Species diversity estimates from Simpson's diversity index for subcortical insects caught in funnel traps by land-area treatment in 2000–2003, Superior National Forest, Cook Co., MN, USA

Year	Cover Type	Undisturbed	Wind-disturbed	Salvaged	Burned
2000	Aspen/Birch/Conifer	0.582	0.528	0.742	NA[a]
	Jack Pine	0.679	0.684	0.837	NA[a]
2001–2003	Jack Pine	0.888	0.912	0.884	0.887

[a] NA = Not Applicable

unexpected in the wind-disturbed and burned areas where large numbers of windthrown trees with exposed roots were present. This suggests that either the rhizophagous beetles persisted at much lower populations than the stem-colonizing beetles in these forests, or that the baited pitfall traps that we used may not have been the optimal trapping method. In 2000, a high release-rate device was used, and the solution evaporated within a week; in 2001–2002, we used a plastic release device from which ethanol did not elute rapidly (David Wakarchuk, Synergy Semiochemical, personal communication); and in 2003, we used the same plastic release device with a hole in the cap. None of these release devices, especially the one in 2003, seemed to attract large numbers of rhizophagous beetles. In the 2000 field season, which occurred one-year post disturbance and had the highest probability of high populations of rhizophagous insects, the high release from the devices may have rendered our traps effectively unbaited through the latter part of the sampling periods. Nonetheless, other workers have found that sampling rhizophagous insects is problematic; by using baited pitfall traps, Erbilgin et al. (2001) caught only 315 beetles in Wisconsin as compared to 2,624 beetles in Louisiana. They further reported that the baited pitfall traps were more effective than lower stem flight traps (Klepzig et al. 1991) in capturing rhizophagous beetles in Wisconsin than in Louisiana. There appear to be regional differences in both the population levels and responses of rhizophagous beetles to trap-type, which might further affect techniques for monitoring in sub-boreal forests. Rhizophagous beetles generally are difficult to sample and observe in the forest due to their cryptic life-stages. Their larvae are associated with roots and adults hide in the forest litter layer (Lynch 1984; Drooz 1985). Other possible methods to sample rhizophagous beetles include the use of insect emergence cages for infested roots, bark peeling of roots, and placement of pitfall traps directly in and around downed trees.

Effects of Semiochemical Treatments on Subcortical Insects

Overall Variability The effects of semiochemical treatments on subcortical insect flight responses generally were dependent upon the year of sampling and land-area treatments. Such high variability may be a reflection of the rapid turnover of subcortical insect communities within the disturbed habitats, and the associated large volume of semiochemical signaling by numerous species present at any given time. For example, semiochemicals of some species of scolytid beetles may interfere with the behavioral responses of related and/or competing species (e.g., allomones) (Wood et al. 1967; Birch and Wood 1975; Furniss and Livingston 1979; Borden et al. 1992; Miller and Borden 1992). Other possible reasons for the variable responses include: 1) high population levels of dominant subcortical species at the time of sampling (*see* next paragraph); and 2) the effects of local physical factors such as forest structure and weather conditions, whose effects on responses to semiochemicals are less well understood (Thistle et al. 2004). For studies conducted within a single habitat and over a short period of time, there is the potential to underestimate the variability of the responses of subcortical insects to semiochemicals.

Subcortical Insect Trap Catches Total catches of all subcortical insects were highest in response to the *D. simplex* bait, especially in 2002 in the burned areas. This trend may reflect high population levels of *D. simplex*, and

Table 5 Species diversity estimates from Simpson's diversity index for subcortical insects caught in funnel traps by semiochemical treatment in 2000–2003, Superior National Forest, Cook Co., MN, USA

Semiochemical Baits	Simpson's index
Dryocoetes spp.	0.935
Dendroctonus valens	0.929
Dendroctonus valens-Phero Tech	0.920
Woodborers	0.919
Unbaited	0.915
Ips perroti-(+)-ipsdienol	0.904
Ips grandicollis	0.873
Ips perturbatus	0.731
Dendroctonus rufipennis	0.640
Ips pini	0.613
Ips perroti-(−)-ipsdienol	0.583
Dendroctonus simplex	0.165

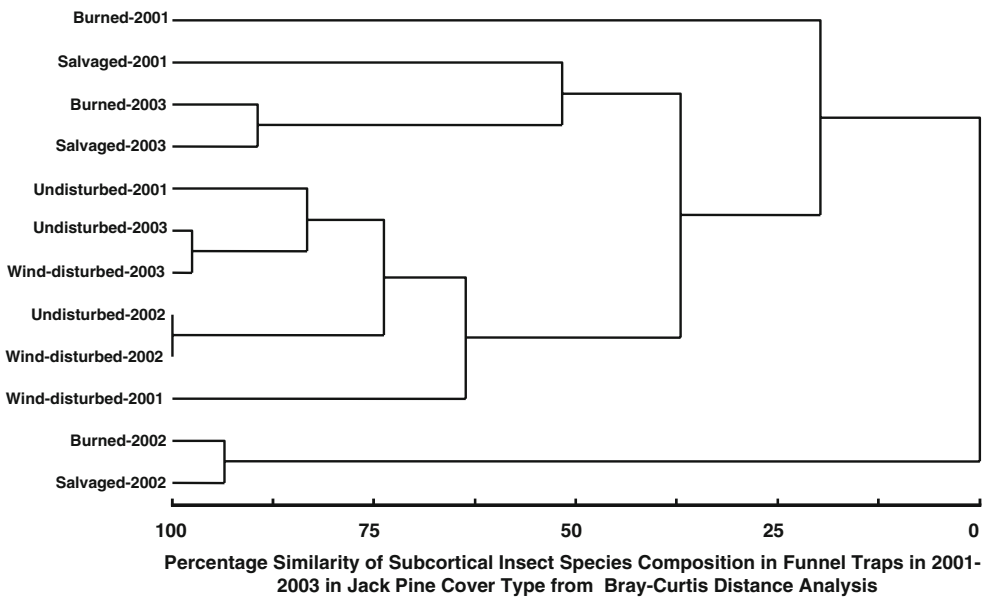


Fig. 6 Dendrogram of the percentage similarity of subcortical insect species trapped in funnel traps in 2001–2003 in the undisturbed, wind-disturbed, salvaged, and burned plots in the jack pine cover type

may account for a delayed response observed for all scolytid beetles. The greatest numbers of scolytids were also caught in response to the *D. simplex* bait in 2002 in the burned areas. This overall trend was driven by catches of *D. simplex*, which was the primary scolytid caught during this field season, and is the only native bark beetle that colonizes and kills tamarack and exotic larches in North America (Langor and Raske 1987; Seybold et al. 2002). In recent years, this species has shown an increase in activity, causing >75% mortality of tamarack trees in some areas of northern Minnesota (Albers 2005).

More predatory checkered beetles (Cleridae) were caught in response to the *D. rufipennis* bait than to the rest of the baits. This was true especially in 2001 in the wind-disturbed forests. The dominant clerid was *T. dubius*, a major predator of *I. pini* in the Great Lakes region (Schenk and Benjamin 1969), and of southern pine beetle, *Dendroctonus frontalis* Zimmermann, in the southern U.S. (Thatcher and Pickard 1966; Berisford 1980). The *D. rufipennis* bait initially was employed in 2000 and 2001 in anticipation of potential population increases of *D. rufipennis*, whose western North American populations respond to wind disturbances (Nelson 1950; Graham 1952; Schmid and Frye 1977; Holsten et al. 1999; Gandhi et al. 2007). However, the commercially available *D. rufipennis* bait was ineffective for attracting eastern populations of *D. rufipennis* in this study and in other studies in northern Minnesota (Seybold et al., unpublished data). We also

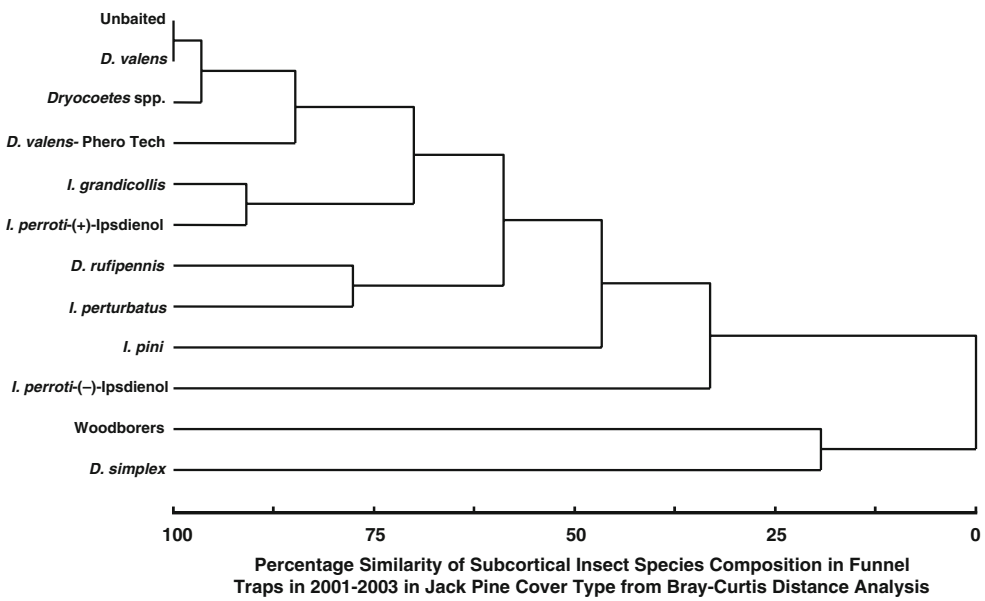


Fig. 7 Dendrogram of the percentage similarity of subcortical insect species trapped in funnel traps in 2001–2003 in response to eleven semiochemicals and an unbaited control trap in the jack pine cover type

caught more individuals of *D. simplex* than of *D. rufipennis* in response to this bait in 2001, and thus, we used the *D. simplex* bait in the subsequent years of the study. Mizell et al. (1984) found that *T. dubius* responded positively to frontalin, ipsdienol, and α-pinene. Vité and Williamson (1970) and Dixon and Payne (1980) also found that *T. dubius* was attracted to frontalin, an aggregation pheromone of *D. frontalis*. Similar results were found by Haberkern and Raffa (2003), where *T. dubius* and *Enoclerus nigrifrons* (Say) were attracted to the commercial *D. rufipennis* bait deployed in traps in *P. resinosa* stands in Wisconsin. This suggests that *T. dubius* is a generalist predator of scolytid beetles in both *Pinus* and *Picea* stands.

As expected, *I. pini* was caught in greater numbers in the *I. pini*-pheromone-baited traps, especially in the year 2002. There is geographic variation in the production and response of populations of *I. pini* to various enantiomeric blends of ipsdienol (Lanier et al. 1980; Birch et al. 1980; Seybold et al. 1995). In western populations, *I. pini* is attracted to 97% (−)-ipsdienol and is inhibited by (+)-ipsdienol (Birch et al. 1980). However, in eastern populations, *I. pini* is attracted to a racemic mixture of ipsdienol (Lanier et al. 1980). The responses of *I. pini* to the enantiomers of ipsdienol have been reported from other North American locations (Raffa and Klepzig 1989; Herms et al. 1991). In northern Minnesota, (+/−)-ipsdienol along with lanierone would be an effective pheromone to monitor the populations of *I. pini* (Teale et al. 1991; Seybold et al. 1992, 1995; Miller et al. 1997).

In 2000, we targeted *I. perroti* by using the combination of (+)-ipsdienol and (−)-ipsenol. However, this bait caught very few *I. perroti*. In 2001–2003, we also tested the combination of (−)-ipsdienol and (−)-ipsenol to target this species. Nearly twice as many *I. perroti* were caught in response to this combination, especially in the undisturbed and wind-disturbed forests. Ayres et al. (2001) reported that populations of *I. perroti* in Wisconsin red pine (*P. resinosa*) forests were attracted to a racemic mixture of ipsdienol and ipsenol. We tested only the pure enantiomers of ipsdienol (and not the racemate) in combination with ipsenol. Our results suggest that (−)-ipsdienol and (−)-ipsenol are effective baits for monitoring *I. perroti* in northern Minnesota.

In 2000, we used *endo*-brevicomin as an attractant to target species of bark beetles in the genus *Dryocoetes*, but we caught only a few specimens of *Dryocoete*s spp. Therefore, in 2001–2003, we used racemic *exo*-brevicomin and (−)-α-pinene (Camacho et al. 1998). No previous research had been conducted on *Dryocoete*s spp. pheromone biology in eastern North America, so we used these western model systems as our best-guess strategy for monitoring biodiversity. Although there was some attraction of *Dr. autographus* to the bait with *exo*-brevicomin, the greatest response by this species was to the woodboring beetle bait, especially in the wind-disturbed forests. Williams and Borden (2004) reported that *Dr. autographus* in British Columbia was most attracted to racemic and (+)-*exo*-brevicomin. Kohnle and Vité (1984) also found that European populations of *Dr. autographus* produce *exo*- and *endo*-brevicomin. The only semiochemical component common between the *Dryocoetes* spp. and woodboring beetle bait was (−)-α-pinene, which was released at an 18-fold higher rate in the latter bait. Camacho et al. (1998) reported similar results for *Dr. confusus* Swaine where (−)-α-pinene improved the response to *exo*-brevicomin in the field. (−)-α-Pinene may be more attractive to *Dr. autographus* than *exo*- or *endo*-brevicomin in our eastern sub-boreal populations.

In 2001–2003, more *Hylurgops r. pinifex* (Scolytidae) were caught in the *D. valens*-Phero Tech and woodboring beetle baits, primarily in the undisturbed and wind-disturbed forests. Very little information is available on the chemical ecology of this species. The common component between *D. valens*-Phero Tech and the woodboring beetle bait is α-pinene. The European species, *H. palliatus* Gyllenhall, was more attracted to ethanol than to host monoterpenes including (+/−)-α-pinene, (+)-3-carene, and terpinolene (Volz 1988; Byers 1992). In contrast, Schroeder and Lindelow (1989) reported that *H. palliatus* was attracted to the combination of both ethanol and α-pinene. Thus, α-pinene may be one of the attractant components for the species in the genus *Hylurgops*.

Among the most abundant subcortical beetles, *B. maculativentris*, *C. virginiensis*, and *P. rufipennis* did not show any response to semiochemical treatments. Both *B. maculativentris* and *C. virginiensis* (buprestid species) may respond to the general odors produced by dead and dying tree hosts (see also Crook et al. 2008). However, Chénier and Philogène (1989) reported a lack of response of buprestid beetles to ethanol and monoterpenes in central Ontario, and they suggest that visual cues may be more important in finding hosts. Similary, Montgomery and Wargo (1983) reported a lack of response of buprestid beetles to ethanol in Connecticut. Male *P. rufipennis* (Scolytidae) produces an aggregation pheromone while feeding on *Picea glauca* trees in British Columbia (Bowers and Borden 1990), identified as 3-methyl-3-buten-1-ol (Bowers et al. 1991). *Polygraphus rufipennis* also was attracted to a *Dryocoetes* spp. bait in *P. resinosa* stands in Wisconsin (Haberkern and Raffa 2003). In our study, it was caught in the second highest numbers in the *Dryocoetes* spp. bait, but in similar numbers in the unbaited funnel trap. These disparate regional results suggest further work on the semiochemicals of *P. rufipennis* is needed.

Overall, attractants for *I. perroti*, *I. pini*, and *D. simplex* were the most useful baits in trapping their respective target

beetles. *Ips grandicollis* and *I. perturbatus* also responded to their respective target baits at a higher level than to any other semiochemical treatments, but *I. grandicollis* and *T. dubius* tended to be semiochemical generalists when compared with the responses of *I. perroti* and *I. pini* to the spectrum of semiochemicals that we tested (Fig. 2). The *D. valens*, *D. valens*-Phero Tech, *Dryocoetes* spp., and woodboring beetle baits were the least efficient in capturing their target beetle species in the sub-boreal forests. *Dendroctonus valens* does not have a known pheromone but it is attracted to (+)-α-pinene, (−)-β-pinene, and (+)-3-carene (host kairomones) in western populations (Hobson et al. 1993; White and Hobson 1993), and to (−)-α-pinene, ipsdienol, and lanierone in eastern populations (Erbilgin and Raffa 2000). We did not find a similar attraction of *D. valens* to ipsdienol and lanierone, thus suggesting that more research needs to be conducted on the semiochemical attractants for this species.

Some species of cerambycid beetles showed a trend of greater attraction to the woodboring beetle bait. Cerambycid beetles have a general attraction to host kairomones and scolytid pheromones, although some short-range sex-pheromones also have been isolated (Allison et al. 2004). The presence of higher concentrations of plant volatiles present in the disturbed forest stands may have interrupted attraction of some beetle species to their respective baits or provided competing sources of attraction. Subcortical species with a primary attraction to host attractants may be difficult to trap and monitor with baited funnel traps in disturbed forests due to olfactory competition with large amounts of damaged and moribund trees.

Subcortical Insect Richness and Diversity The greatest diversity of subcortical insect was observed in response to the *D. valens* and *Dryocoetes* spp. baits. Both of these baits had monoterpenes that may have acted as a general attractant for subcortical insects. It also is likely that other bait types with pheromone components may have deterred non-target subcortical insect species. *Dendroctonus valens* and *Dryocoetes* spp. baits, although not as effective in capturing their target species, may have greater efficacy in capturing a broader spectrum of subcortical species in a sub-boreal forest. The lowest biodiversity was recorded from traps baited with lures for *I. pini, I. perroti*, and *D. simplex*, perhaps reflecting the specificity of these baits.

Subcortical Insect Species Composition Cluster analyses of subcortical insect species composition among the semiochemical baits in 2001–2003 revealed that woodborer and *D. simplex* baits elicited the most distinct species composition (there was only 2% similarity with the rest of the bait types). Such differences could be attributed to the efficacy of the woodborer and *D. simplex* baits in attracting their target beetles, and their associated subcortical insects (Table 5). This result also indicates that subcortical insect species that responded to the woodborer and *D. simplex* baits may differ from those that responded other bait types. The species composition of subcortical insects that responded to the *I. grandicollis* and *I. perroti* baits clustered together with 85% similarity. As these baits had (−)-ipsenol in common, this pheromone component may have the capacity to attract similar subcortical species. The species assemblages that responded to the *D. valens* and *Dryocoetes* spp. baits, and the unbaited traps clustered together with >85% similarity, which suggests that the *D. valens*- and *Dryocoetes* spp.-baited traps acted almost like unbaited funnel traps. These two baits and the unbaited trap caught both the greatest numbers of similar, though not identical, subcortical species.

Effects of Land-Area Treatments on Subcortical Insects

Subcortical Insect Trap Catches From 2000–2003, total insect catches between the wind-disturbed and undisturbed forest plots generally were similar. However, in 2001 we caught approximately 2.5-fold more subcortical insects in the wind-disturbed than in the undisturbed areas. Thus, the trap catches of subcortical insects were high 2 years after the wind storm (in 2001), but then they declined. Possible reasons for the decline of early-successional subcortical insect flight activity with time are: increasing populations of insect (Ryall 2003; Ryall et al. 2006) and avian (Baldwin 1968; Fayt et al. 2005) natural enemies (Ryall and Smith 2001); greater inter- and intra-specific competition (Robins and Reid 1997); lower availability and quality of CWD as habitat on the disturbed landscape (Wallace 1953; Wickman 1965; Jacobs 2004); and/or rapid salvaging of other downed material on the landscape (USDA Forest Service 1962, 1965). Similar results were reported for the activity of *I. pini* in ice-damaged *P. resinosa* stands in Ontario where beetle activity declined markedly 2–4 years after the storm (Ryall et al. 2006), and for the activity of *Dendroctonus brevicomis* LeConte in wind-damaged stands in California where beetle activity declined 2 years after the storm (Miller 1928). Populations of *Ips typographus* (L.) and other subcortical beetles peaked 2 years after a wind storm (Vivian) in Switzerland (Wermelinger et al. 1999) and after another wind storm (Lothar) in 1999 in France (Nageleisen 2001). In contrast, after the storm in France, ambrosia beetles (Scolytidae) in the windthrow gaps of a hardwood forest did not increase subsequently in abundance when compared to undisturbed forest areas (Bouget and Noblecourt 2005). Thus, responses of subcortical beetles to wind storms may be taxon specific, and may vary across forest types.

In 2001 and 2002, similar numbers of all subcortical insects were caught in funnel traps in the wind-disturbed and burned forests. However, in 2003, the trap catches of

subcortical insects were lower in the salvaged and burned areas as compared to the wind-disturbed sites. This suggests that although burning initially retained similar or higher numbers of insects than the wind-disturbed areas, flight activity of subcortical insects decreased 4 years after the wind storm in the areas where two disturbances were combined. One explanation for this effect is that the wind-disturbed sites continued to provide food for the beetles (i.e., residual and leaning trees continued to die into the fourth year after the storm). In the burned or logged areas, the residual trees did not die (or there were few residual trees). Alternatively, olfactory cues from the burning and/or logging may have attracted subcortical insects into these treatment sites in 2001 and 2002, but perhaps because of an absence or paucity of host material from the treatments, the insects were lured into traps rather than colonizing and reproducing in the woody debris. Werner (2002) reported that in *P. glauca* stands in Alaska, both clear-cutting and burning increased the populations of subcortical scolytid, buprestid, and cerambycid beetles during the first year after the land-area treatments. Populations of these Alaskan subcortical beetles declined to the pre-disturbance level 5–10 years later. In our study, the decline following the disturbances may have occurred on an even shorter time scale. In black spruce, *Picea mariana*, stands in Quebec, Boulanger and Sirois (2007) described two phases of subcortical insect colonization of trees after burning. The first phase occurs shortly (up to 1 year) after tree death from the fire. Insect populations decline for several years after this phase, but then increase again during the second phase when dead trees fall to the ground and are colonized by fungivorous and saprophagous subcortical insects. Our study likely did not continue long enough to capture this second phase of population increase, nor did we attempt to capture saprophages.

At the family level, flight-activity responses of subcortical insects to forest disturbances also varied with the year of sampling. In 2002, scolytid beetles responded in greater numbers in salvaged and burned as compared to undisturbed forest areas. In contrast, in 2001, clerid and buprestid beetles responded in greater numbers in all land-area treatments as compared to undisturbed forest areas. We hypothesize that the response of scolytid populations showed a lag-period because they may have originated and slowly increased in numbers from within the disturbed stands (Wermelinger et al. 2002). We also observed limited colonization of downed trees in summer 1999 and spring 2000 by scolytids (K.J.K.G. and S.J.S., personal observations). Buprestid beetles in the disturbed stands may have originated from immigration from other stands as a response to the increased amounts of CWD, and/or from within the stand as they generally take 2 years to develop (Bright 1987). Greater populations of clerid beetles present in 2001 likely were due to their strong attraction to frontalin in the *D. rufipennis* bait, which was used only in this year, and to the increased prey populations after the disturbance event (see next paragraph).

At the species level, in 2001, *I. pini*, *T. dubius*, and *M. s. scutellatus* increased their flight activity in the wind-disturbed and burned areas, and *B. maculativentris*, *T. nubilus,* and *P. rufipennis* showed similar flight activity in the burned areas. However, abundance declined 4 years after the storm. Thus, these species responded positively for a short-time to a compound disturbance on the landscape (i.e., wind followed by burning). In contrast, other species such as *C. virginiensis* and *Dr. autographus* did not increase in numbers in either the wind-disturbed or the burned areas, indicating that these species either do not always respond to the increased amounts of CWD on the landscape or that there may be greater negative pressure from competition, predators, and parasites in disturbed stands.

Among the Scolytidae, relatively more males of *I. perroti* were caught in 2001 than in 2002–2003, whereas for *I. grandicollis* relatively more males were caught in 2003 than in 2001–2002. In 2001, a greater percentage of male *I. perroti* were caught in the burned forests than in the rest of the land-area treatments. We found similar numbers of male and female *I. perroti* emerging from *P. banksiana* trees (Gandhi 2005), so we expected an equal sex-ratio in the flight response of *I. perroti* to the semiochemical-baited traps. Possible reasons for a skewed sex ratio include differential mortality of the sexes (Cameron and Borden 1967) (across land-area treatments and years) or a differential attraction of the sexes to traps in the various land-area treatments.

Subcortical Insect Richness and Diversity In Minnesota there were reportedly 23 species of buprestids (Downie and Arnett 1996), 275 species of cerambycids (Downie and Arnett 1996; Yanega 1996), one species of histerid (Downie and Arnett 1996), 64 species of scolytids (Dodge 1938; Wood 1982b), and 4 species of siricids (Smith and Schiff 2002). Our field study and concurrent museum survey established new Minnesota state records for 23 subcortical insect species in six families: *C. sexsignata*, *C. striata*, *C. sulcicollis*, *P. aeneola*, and *P. d. drummondi* (Buprestidae); *P. n. nigrella* and *X. tibialis* (Cerambycidae); *E. muttkowski*, *E. n. rufiventris*, *M. dislocatus*, *T. trifasciatus*, *T. nubilus, T. u. undatulus*, *T. nutalli*, and *Z. sanguineus* (Cleridae); *P. teres*, *P. coarctatum*, *P. deficiens*, and *P. lecontei* (Histeridae); 5) *I. latidens* (Scolytidae); and *S. behrensii*, *U. g. flavicornis*, and *X. spectrum* (Siricidae). The University of Minnesota Insect Collection specimens of previously unreported subcortical species in Minnesota were collected as early as 1894, and in the case of *T. nutalli*, were distributed in as many as 21 counties. In most cases, these specimens represented native fauna from local vegetation. In our field

study, the taxa were also derived primarily from the native fauna present in the sub-boreal Minnesotan forests. However, both *P. d. drummondi* and *P. n. nigrella* have been reported recently from western larch (*Larix occidentalis* Nutt.) logs imported to central Minnesota from Montana (Dodds et al. 2004). Regional distributions of most insects we collected suggest they are native in northeastern Minnesota. For example, *Chrysobothris sexsignata*, *E. n. rufiventris*, and *T. nutalli* occur in *P. resinosa*, *P. banksiana*, and *P. strobus* stands in the adjacent province of Ontario, Canada (Chénier and Philogène 1989), and *I. latidens* occurs in Ontario in *P. strobus* (Bright 1976). *Enoclerus muttkowski*, *E. nigripes*, *T. undatulus*, and *Z. sanguineus* occur in stands of *P. resinosa* in the adjacent state of Wisconsin (Aukema and Raffa 2000, 2005; Aukema et al. 2000a, b, 2004, 2005; Erbilgin and Raffa 2001; Erbilgin et al. 2003) and *Z. sanguineus* may be associated with a hardwood tree (butternut), *J. cinera*, in Wisconsin (Katovich and Ostry 1998).

Sirex behrensii is a woodwasp primarily found in western North America, but it was recently collected in Ohio, where it was found emerging from imported lumber (Smith and Schiff 2002; D. Smith, personal communication). Hence, either the range of this species is more extensive than previously thought, or it has also been introduced to the forests of Minnesota. It is important to note that this species erroneously has been reported to occur in Virginia and Florida (Smith and Schiff 2002: 178; D. Smith, personal communication).

In 2001–2003, the wind-disturbed forest areas in Minnesota had greater species richness and diversity than the undisturbed areas. Wind storms provide a sudden and localized increase in CWD, which may provide habitat for subcortical insect species (Bouget and Duelli 2004; Gandhi et al. 2007). After windstorm Vivian in Switzerland, there was also an increase in the species richness of scolytid and cerambycid beetles in wind-disturbed areas as compared to the cleared wind-disturbed sites (Wermelinger et al. 2002, 2003). In our study, species richness and diversity were greater in burned areas than in salvaged areas, which suggests that greater numbers of beetle species were attracted to the recently burned, larger-sized pieces of CWD (Gilmore et al. 2003), and to fire-damaged and dead residual trees. There is evidence that fire-damaged trees produce greater amounts of ethanol and, therefore, attract greater numbers of subcortical beetles than do undamaged trees (Santoro et al. 2000; Kelsey and Joseph 2003). Although burning eventually results in lowered population levels of early-successional subcortical beetles, it may actually result in a greater species richness and diversity on the sub-boreal landscape (Santoro et al. 2000). It presently is unclear whether prescribed burning will retain similar species diversity to that of naturally burned forests, as there may be inherent differences in the amounts and types of CWD in the two disturbed habitats.

Subcortical Insect Composition Analysis of our data from 2001–2003 on species composition indicated that the assemblages from undisturbed and wind-disturbed forests clustered together, whereas the assemblages from salvaged and burned forests also clustered together. Although the wind-disturbance event created regional habitats for species diversity, as has been reported for European forests (Wermelinger et al. 2002, 2003), there is a closer resemblance of the species composition of the undisturbed to the wind-disturbed forest (Bouget and Noblecourt 2005), and of the salvaged to the burned forest. This suggests that the salvaged and burned forests are being colonized by species of subcortical insects different from those that colonize the undisturbed and wind-disturbed forests. Such trends also could be attributed to the similar types (e.g., leaning trees), though different amounts, of CWD present in the undisturbed and wind-disturbed forests, and to the compounded disturbances in the salvaged and burned forests. As in the case with ground beetle assemblages (Gandhi 2005; Gandhi et al. 2008), the fuel-reduction treatments after a wind-disturbance event may have the potential to alter succession of subcortical insects. At present, it is unclear whether the species compositions of the subcortical insects in undisturbed and wind-disturbed forests will become similar to those of the salvaged and burned forests with time; longer term sampling would be necessary to address this question.

Conclusions

In summary, in 2000, we trapped one-third more subcortical insects in jack pine than in aspen/birch/conifer stands. In 2001–2003, we trapped only in the jack pine cover type, and the insect responses to land-area treatments (wind disturbance, salvage-logging, and prescribed-burning) varied with year of sampling and semiochemical treatment. Trap catches of all subcortical insects were three-times greater in 2001–2002 than in 2003, suggesting that these insects were most abundant in the wind-disturbed areas 2 years after the storm event, and then declined. In 2001, approximately 2.5-fold more subcortical insects of all taxa were trapped in wind-disturbed areas than in undisturbed areas. In 2001–2002, trap catches of beetles such as *I. pini*, *M. scutellatus scutellatus*, and *T. dubius* were similar in the wind-disturbed and burned forests. In 2003, catches of these species in the burned forests were reduced in half relative to prior years.

In 2000, salvaged forest areas had the highest species richness and diversity, whereas in 2001–2003, wind-

disturbed forest areas had the highest species richness and diversity. Both wind disturbance and burning increased the subcortical insect species richness and diversity on the landscape. We provide new state records for 23 subcortical insect species in Minnesota, which underscores the efficacy of semiochemicals as tools for determining regional biodiversity. Species compositions of subcortical insects in the undisturbed and wind-disturbed forests were distinct from those of the salvaged and burned forests. Although prescribed-burning could be an effective tool to increase species diversity in the landscape, it could result in different species composition over time.

Trap catches of subcortical insects in response to semiochemical treatments also varied with year of sampling and land-area treatment. Such variability with year has been reported before in the case of *I. pini* and *T. dubius* (see Raffa and Klepzig 1989 vs. Herms et al. 1991). During 2002, relatively high population levels of *D. simplex* dominated the response of all subcortical insects as its attractant [(+/−)-seudenol and (−)-α-pinene] elicited catches of four- to five-times more insects than the other treatments. In general, the predaceous checkered beetle, *T. dubius*, responded the most to traps baited with the attractant for *D. rufipennis* [(+/−)-frontalin, (+/−)-1-methyl-2-cyclohexenol, and (−)-α-pinene]; *I. perroti* responded most to (−)-ipsdienol and (−)-ipsenol; *I. pini* the most to (+/−)-ipsdienol and lanierone; *Dr. autographus* the most to a woodborer (i.e., buprestid, cerambycid, and siricid) attractant [ethanol and (−)-α-pinene]; and *H. rugipennis pinifex* mostly to the Phero Tech *D. valens* [(+)-α-pinene, (−)-β-pinene, and (+)-3-carene] and woodborer attractants. The greatest diversity of subcortical beetle species was in traps baited with attractants for *D. valens* [(+)-α-pinene and (−)-β-pinene] and *Dryocoetes* spp. [*exo*-brevicomin and (−)-α-pinene] perhaps reflecting the generic nature of the baits. The most distinct species compositions were collected in response to the woodborer and *D. simplex* baits, whereas the species compositions in traps with the *D. valens* and *Dryocoetes* spp. baits, and the unbaited funnel trap were the most similar. Our 4-year study across various landscapes suggests that the responses of subcortical insects to semiochemicals are more complex than previously appreciated, and that future researchers should anticipate species variation in space and time.

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known presence of moths, eggs or larvae (P. Walker unpublished data).

Polyene hydrocarbons and their epoxides constitute a second major class of lepidopteran sex pheromones that have been isolated from four major families: Geometridae, Noctuidae, Arctiidae, and Lymantriidae (Millar 2000; El-Sayed 2008). To date, C19 triene has been identified as an attractant or pheromone component in 34 other species of Geometridae, 24 of which belong to the same subfamily as *M. privata* (Ennominae) (El-Sayed 2008). In all species that use C19 triene, except *M. privata*, other bioactive compounds also have been found in pheromone extracts. The use of two or more components in a pheromone is often crucial in maintaining a discrete, species-specific chemical communication channel (Cardé and Baker 1984; Millar et al. 1990). Although *M. privata* is the only Australian geometrid in which C19 triene has been verified as a sex pheromone component, Steinbauer et al. (2004) gave indirect evidence that this compound is used for communication by other sympatric species as three species of Ennominae (two belonging to the same genus) also were caught during field trials. Therefore, it is likely that additional pheromone components are used by *M. privata*. Steinbauer et al. (2004) detected two alcohols (1-hexadecanol and 1-octadecanol) in some female *M. privata* extracts, but neither compound elicited significant antennal responses in males, nor did their addition to lures containing C19 triene enhance trap captures.

The purpose of this study was to search for other pheromone components in female *M. privata*. The response of male *M. privata* to a previously unidentified pheromone component was measured in the laboratory by an electroantennogram, and in the field by using pheromone traps in *Eucalyptus* spp. plantations in Tasmania, Western Australia, Victoria, and South Australia. We also determined the optimal ratio of the two synthetic pheromone components for attraction of male *M. privata*, compared the trapping efficacy of two types of traps, examined the effect of lure age on trap catch, and, for comparison, analyzed male *M. privata* abdominal tip extracts.

Methods and Materials

Insects A colony of *M. privata* was founded (21°C±1°C; 60–70% RH; L12: D12 photoperiod) from collections of eggs and early instar larvae infesting *Eucalyptus globulus* trees near Cornelian Bay, Hobart, Tasmania, during April and May 2005. Larvae were reared on juvenile *E. globulus* leaves placed in ventilated plastic boxes lined with paper toweling and, at the prepupal stage, were placed in boxes containing vermiculite for pupation. Pupae were sexed according the position of the genital scar, stored separately, and monitored daily for emergence. Upon emergence, moths were placed in individual 850-ml round plastic containers (Genfac Plastics Ltd, Melbourne, Australia) with a honey/sugar solution (20 g honey, 20 g sugar, and 2 g ascorbic acid in 1 l boiled water) and held until experimentation.

Extracts Abdomen tips containing the ovipositor and associated pheromone gland were excised from unmated, laboratory-reared, *M. privata* females, 1 (i.e., newly emerged), 3, 5, 7, and 9-d-old (N=10, 8, 10, and 5, respectively), and from three mated females of unknown age. Tips were removed during the first 2 h of the scotophase to increase the chance of sampling calling females. The tips from ten female *M. privata* caught in a light trap 50 km east of Albany, Western Australia, also were analyzed. After freezing females and removing the abdominal tips, each was dissected to determine its mating status. Tips from all females were placed into individual borosilicate vials with 150 µl inserts (Waters Corporation, Australia), soaked in 15 µl of CH_2Cl_2 containing 100 ng methyl stearate as an internal standard for an hour at room temperature, and then the tips were removed before storing the extract at −20°C. The solutions were allowed to reach room temperature before analysis.

Abdomen tips (the genitalia, claspers, and surrounding tissue) were excised from laboratory-reared male *M. privata* moths 1, 3, and 5-d-old (N=3 per age category), and soaked in CH_2Cl_2 as described above. All males were unmated, and had no access to females.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis GC-MS analyses were carried out on a Varian 3800 GC coupled to a Varian 1200 triple quadrupole MS using 70 eV electron ionization in single quadrupole mode. The column was a Varian 'Factor Four' VF-5 ms (30 m×0.25 mm internal diam and 0.25 micron film). Injections of 1 µl of abdominal tip extracts were made either manually or with a Varian CP-8400 autosampler in conjunction with a Varian 1177 split/splitless injector at 250°C in the splitless mode. The ion source was held at 220°C, and the transfer line at 290°C. The carrier gas was helium at 1.22 ml/min using a constant flow mode. The GC oven was held at 60°C for 1 min then ramped to 150°C at 30°/min, then to 280°C at 8°/min.

Identities of major compounds were assigned based on the previous report from Steinbauer et al. (2004) on this species, mass spectra, and Kovats retention indices relative to those reported in the NIST Chemistry WebBook (http://webbook.nist.gov/chemistry/). Lower levels of trienes were detected in moth extracts by selected ion monitoring (SIM) using characteristic ions at m/z 79 and 108, which were used for comparison of C19 and C21 triene levels. Levels of trienes were estimated in each moth from the ratio of the

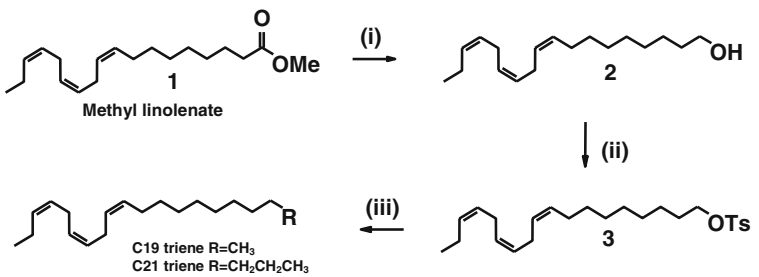
response in full scan mode of the triene to the methyl stearate internal standard.

Solutions of C19 and C21 trienes for EAG and field testing were prepared in various C19/C21 proportions ranging from 3:2 to 67:1. These ratios were independently verified by GC-MS analysis of an aliquot of each solution.

Synthesis (3*Z*,6*Z*,9*Z*)-3,6,9-Nonadecatriene (>98% purity) was prepared by JAS and PPM, at the University of Tasmania, as described by Davies et al. (2007). Synthesis of (3*Z*,6*Z*,9*Z*)-3,6,9-henicosatriene (>99.7% purity) was conducted by AN, FA and EH, at Mid Sweden University, as shown in Scheme 1. Unless otherwise stated, starting materials and solvents were used as received from commercial suppliers. Dry THF (benzophenone and potassium) and Et_2O ($LiAlH_4$) were distilled from the indicated drying agents and either used immediately or stored under argon. NMR spectra were recorded on a Varian 500 instrument. GC analyses were carried out using a 30 m×0.25 mm id capillary column coated with EC-1, d_f=0.25 μm, carrier gas: N_2 (15 psi) and a split ratio of 1:20. Mass spectra were recorded on a Varian Saturn 2000 GC-MS instrument. Silica gel 60 (230–400 mesh; Merck, Sigma-Aldrich, Scandinavia) was used in preparative liquid chromatography. Thin layer chromatography (TLC) was performed on silica gel plates (Merck 60, pre-coated aluminium foil) eluted with EtOAc in cyclohexane (40%).

*(9Z,12Z,15Z)-9,12,15-Octadecatrien-1-ol (***2***)* Methyl linolenate (**1**) (1.08 g, 3.69 mmol) dissolved in Et_2O (5 ml) was added to a suspension of $LiAlH_4$ (295 mg, 7.39 mmol) in Et_2O (20 ml) at 0°C, under an argon atmosphere. After 1.75 h, the reaction was quenched with 2 M HCl (aqueous, 15 ml) at 0°C. The organic layer was separated, followed by extraction with Et_2O (3×30 ml), washing of the combined extracts with brine (30 ml), and drying ($MgSO_4$). Evaporation of solvent under reduced pressure resulted in a clear bright yellow oil that was purified by flash chromatography (silica, EtOAc/cyclohexane). Compound **2** was isolated as a clear bright yellow oil, 919 mg (3.48 mmol, 94% yield), with 100% purity according to GC. ^1H and ^{13}C NMR spectra were identical with those reported in literature (Wang and Zhang 2007).

(3Z,6Z,9Z)-Henicosa-3,6,9-triene (C21 triene) 4-Methylbenzene-1-sulfonyl chloride (1.29 g, 6.78 mmol) was added to a solution of alcohol **2** (897 mg, 3.39 mmol) and pyridine (0.80 ml, 10.2 mmol) in CH_2Cl_2 (30 ml) at 0°C, under an argon atmosphere. The reaction mixture was allowed to remain at room temperature for 4 d, after which Et_2O (50 ml) and H_2O (30 ml) were added to the mixture. The organic layer was separated, followed by extraction with Et_2O (3×30 ml), washing of the combined extracts with 2 M HCl (aqueous, 2×30 ml) and brine (2×30 ml), and drying ($MgSO_4$). Evaporation of solvent under reduced pressure resulted in a clear bright yellow oil that was purified by flash chromatography. Tosylate **3** was isolated as a clear, colorless oil, (1.56 g) containing large amounts of 4-methylbenzene-1-sulfonyl chloride. The excess of sulfonyl chloride was removed by reaction of the product mixture with 1,4-butandiol (0.70 ml) in pyridine (1.0 ml) at 5°C overnight. Et_2O (25 ml) and H_2O (25 ml) were added, and the aqueous phase was acidified with 2 M HCl. The organic layer was separated, followed by extraction with Et_2O (4×30 ml), washing of the combined extracts with brine (50 ml), and drying ($MgSO_4$). Evaporation of solvent under reduced pressure resulted in a yellow turbid oil that was purified by flash chromatography. The tosylate **3** was



Tasmania
(i) $LiAlH_4$, THF, 0°C to room temp.
(ii) p-toluenesulfonyl chloride, pyridine, CH_2Cl_2, 0°C
(iii) $(R)_2CuLi$, THF, 0°C

Sweden
(i) $LiAlH_4$, Et_2O, 0°C
(ii) p-toluenesulfonyl chloride, pyridine, CH_2Cl_2, room temp.; 1,4-butanediol, pyridine, 5°C
(iii) RMgBr, CuI, THF, -30°C to room temp.

Scheme 1 Synthetic schemes for (3*Z*,6*Z*,9*Z*)-3,6,9-nonadecatriene (conducted at the University of Tasmania) and (3*Z*,6*Z*,9*Z*)-3,6,9-henicosatriene (conducted at Mid Sweden University)

isolated as a clear colorless oil, 1.07 g (2.56 mmol, 76% yield, pure according to TLC), and used in the next step without further characterization.

Propyl magnesium bromide was prepared by addition of propylbromide (0.47 ml, 5.2 mmol) in THF (5 ml) to magnesium chips (256 mg, 10.5 mmol) under an argon atmosphere. The reaction started spontaneously. After 15 min, the Grignard reagent was transferred by syringe to a flask loaded with CuI (488 mg, 2.56 mmol) at −30°C under an argon atmosphere. After another 15 min, tosylate from above (1.07 g, 2.56 mmol) was added to the grey suspension. The mixture was allowed to reach room temperature slowly and turned black. After 4 d, the reaction was quenched with aqueous saturated NH_4Cl (25 ml) followed by addition of H_2O (25 ml), extraction with Et_2O (3×30 ml), washing of the combined extracts with brine (30 ml), and drying ($MgSO_4$). Evaporation of solvent under reduced pressure resulted in a yellow oil that was purified by flash chromatography. (3*Z*,6*Z*,9*Z*)-Henicosa-3,6,9-triene was isolated as a clear colorless oil, 544 mg (1.87 mmol, 73% yield), with 99.7% purity according to GC. ^{1}H and ^{13}C NMR spectra were identical with those reported in literature (Wang and Zhang 2007). MS (EI) *m/z* (relative intensity): 290 (13 %, M+), 261 (6), 247 (4), 234 (23), 219 (3), 205 (5), 191 (4), 177 (6), 163 (11), 149 (16), 135 (30), 121 (34), 108 (100), 95 (83), 81 (90), 79 (83), 67 (60), 57 (20), 55 (19), 41 (32), 39 (29).

Electrophysiology Electroantennogram (EAG) recordings were prepared by placing an antenna excised from a male between two silver wire electrodes, covered by thin glass capillaries filled with Ringers solution. Antennal responses were recorded via an Intelligent Data Acquisition Controller (IDAC-02, Syntech, Hilversum, the Netherlands) connected to a PC. Antennal preparations were exposed continuously to charcoal-filtered and moistened air with a velocity of 0.5 m/s. Test compounds (5 µl of hexane on filter paper in a Pasteur pipette) were delivered via a 1 s puff of air (5 ml/s) into an opening in the glass tube 150 mm upstream from the antenna, with the outlet 10 mm from the antenna. Stimuli were administered by that device. The solvent was allowed to evaporate from the filter paper prior to testing. The EAG responses of males to the C19 and C21 trienes, hexadecyl acetate, and 1-octadecanol (all >99% purity) were tested on the antennae of four to 12 males. EAG recordings were analyzed using 'GcEad' software, version 4.1 (Syntech, the Netherlands 2005). For data analysis, the mean solvent signal was subtracted from each mean stimulus signal. All amplitudes were normalized to the amplitude recorded in response to the reference stimulus.

GC-Electroantennogram (EAD) analysis that used combined gland extracts from three virgin females was conducted on antennae of males prepared as described above. Extracts (1 µl) were injected into a Varian Vista 6000 GC in splitless mode with an injection temperature of 220°C, and the oven was held at 60°C for 1 min and then ramped to 240°C at 20°/min, with a 5 min hold. The GC was equipped with an HP-5, (5% phenyl)-methylpolysiloxane column, 12 m×0.2 mm id, phase thickness 0.33 ìm. Nitrogen was used as carrier gas. A GC effluent splitter (split ratio 1:1) was used. One stream was directed to a flame ionization detector (FID), and the second was added through a heated transfer line to the air stream and directed over the excised antenna of a male moth, as described above. EAD signals and FID responses were recorded simultaneously by the IDAC2 connected to a PC via Syntech GcEad software.

Field Trials Field trials were carried out in commercial plantations of *Eucalyptus nitens* and *E. globulus*, 2–5-y-old, in Tasmania, Western Australia, Victoria, and South Australia in 2007 and 2008. Lures were prepared by dissolving compounds in hexane, with *ca* 1% of the antioxidant butylated hydroxytoluene, and applying 100 µl of solution into rubber septa (Sigma-Aldrich, Australia), then allowing the solvent to evaporate in a fume hood before storing at −20°C. Control septa were loaded with 100 µl of hexane. Unless otherwise stated, green Unitraps (PheroBank, the Netherlands) were used in trapping studies. The base of each trap was sprayed with a persistent surface insecticide to kill trapped moths and to prevent moths from being removed by ants and wasps. Traps were suspended within tree rows from metal poles at *ca* 1.5 m height.

Experiment 1. Field Bioactivity of C21 triene: Starting in March 2007, captures of male *M. privata* in Unitraps baited with 1 mg, 5 mg, or 10 mg C19 triene and 1 mg C19 triene + 25 µg C21 triene (40:1 ratio) were compared in commercial *Eucalyptus* plantations at various locations in southern Australia. In Tasmania, three replicates of each lure type were placed in 2–5-yr-old *E. nitens* plantations at the Surrey Hills estate, near Hampshire (NW Tasmania), three in Gould's Country (NE Tasmania), three in the Geeveston area and one near Pittwater and one near Nugent (Southern Tasmania). Three replicates of each lure type (except the 5 mg C19 triene dose) were placed in 2–5-yr-old *E. globulus* plantations near Albany, Western Australia, and four replicates were placed in the Green Triangle area on the Victoria/South Australian border. Single lines of Unitraps containing each lure type were placed 10–30 m apart at a height of 1.5 m in random order, spacing replicate rows at least 100 m apart. The position of each lure type within a replicate row was assigned randomly. Traps were checked on a weekly or fortnightly basis, and trap positions within rows were rotated in order to reduce

any positional bias in moth capture. Captured moths were identified, counted, and sexed. In Victoria/South Australia and Western Australia, trapping was terminated in May and June 2007, respectively. In Tasmania, trapping extended to February 2008, and lures were changed in December 2007 as detailed in Experiment 2.

Experiment 2. Optimal Ratio of C19 and C21 Trienes for Trapping Male M. privata: Lures loaded with blends of C19:C21 trienes at 3:2, 16:1, 33:1, 40:1, and 67:1 ratios were made by adding the required amount of C21 triene to 1 mg C19 triene. Lures loaded with 1 mg C19 triene only, 25 μg of C21 triene only (equivalent to the amount of C21 triene added to 40:1 ratio C19:C21 triene baits), and hexane only (controls) also were formulated. Single rows of randomly assigned Unitraps each containing one of the test lures were spaced 10 m apart at 1.5 m height in 2–5-yr-old *E. nitens* plantations at five locations in the Surrey Hills estate. Replicate rows of traps were spaced at least 100 m apart. The number of male *M. privata* caught was monitored 19 December 2007 to 18 March 2008, checking traps and rotating their positions about every 2 wk. Baits were not replaced over the trapping period.

Experiment 3. Comparison of Trap Design: The trapping efficiency of the green Unitraps was compared with white Delta traps in a 4-yr-old *E. nitens* plantation near Buckland, southern Tasmania. Five pairs of each trap design were placed 10 m apart, with a distance of 5 m between pairs and a trap height of 1.5 m. All traps were baited with lures containing 1 mg C19 triene + 60 μg C21 triene (16:1 ratio). The order of trap design within each pair was assigned randomly. Trap catch was checked once after a 4 d trapping period between 4 and 8 April, 2008.

Experiment 4. Effect of Lure Age on Trap Catch: The number of male *M. privata* caught in five Unitraps baited with fresh lures loaded with 1 mg C19 triene + 60 μg C21 triene (16:1 ratio) was compared with the catch for five traps baited with comparable lures that had been aged in the field for 3 mo from experiment 2. Deployment of traps was as for Experiment 3. The order of trap design within each pair was assigned randomly. Trap catch was checked twice over a 24 d trapping period between 8 April and 2 May, 2008, in the *E. nitens* plantation near Buckland, southern Tasmania.

Statistical Analysis SPSS version 16.0 for Windows (SPSS Inc. 2007) was used to analyze EAG and field trapping data. EAG responses to test compounds (mV) were normalized to the mean response to hexane as the reference stimulus (= 100%). Normalized antennal responses and pheromone trapping data from Experiments 1 and 2 were analyzed using ANOVA, while data from Experiments 3 and 4 were compared using *t*-tests. Data was checked for variance homogeneity by using Levene's homogeneity-of-variance test and transformed if necessary. If the ANOVA *F*-value was significant, differences between means were then tested for significance by the least significant difference test (LSD) at the 5% probability level.

Results

GC-MS Analysis of Solvent Extracts Twenty-one compounds were identified tentatively from gland extracts of 41 individual *M. privata* females from Tasmania by GC-MS (Table 1). Detailed examination of the mass spectra of all major and minor compounds in an individual female with relatively high levels of C19 triene revealed one compound (peak 8 in Fig. 1) that had very similar MS characteristics of a polyunsaturated compound (i.e., ions at *m/z* 67, 79, and 108). Also, the diagnostic ion at *m/z* 206 observed in the C19 triene (M- 56) was seen at *m/z* 234, consistent with an extra C_2H_4 unit (Fig. 2). Given that (3*Z*,6*Z*,9*Z*)-3,6,9-henicosatriene already had been recorded as a pheromone component in some Geometridae species (Millar 2000; El-Sayed 2008), this compound was synthesized. The synthetic C21 triene retention characteristics and mass spectrum were indistinguishable from that of the moths' compound, and the standard co-eluted with female moth component 8. Other components of interest found in female extracts were hexadecyl acetate along with the previously reported *n*-hexadecanol and *n*-octadecanol (Steinbauer et al. 2004). These three compounds were found in only a few females in variable quantities. Levels of trienes also were extremely low in some moths, and it was not possible (even by SIM-MS) to determine the C19/C21 triene ratio in all individuals. The C21 triene was below the detection limit in 23 out of 41 extracts, while the C19 triene was below detection limit in one female extract. Consequently, the calculated C19:C21 ratio is based on 15 individual females of known age and three of unknown age in which both compounds could be measured. Mean estimated titers of trienes per female (calculated as internal standard equivalents) were 30.74 ng (± 6.1 SE) (range= 604 pg to 143 ng) for C19 triene, and 1.44 ng (± 0.3 SE) (range=368 pg to 3.8 ng) for C21 triene. Estimated titers of C19 and C21 trienes were highly correlated (Pearson's correlation coefficient=0.956, $P<0.001$, $N=15$) (Fig. 3a). Mean titers of both compounds peaked in 7-d-old females and decreased markedly in 9-d-old females (Fig. 3b), but was again highly variable within age categories. The mean ratio of C19:C21 trienes in female *M. privata* from Tasmania was 33:1 (± 2.3 SE, range 8:1 to 54:1), compared to females from Western Australia which exhibited a mean C19:C21 trienes ratio of 5.7:1 (± 0.6 SE, range 3.5:1 to

Table 1 Peak number, kovats retention index and tentative. identities of the main compounds detected in abdominal tip extracts from female *Mnesampela privata*

Peak number[a]	Kovats index (on VF-5ms)	Compound
1	1825	*n*-hexadecanal
2	1876	(3*Z*,6*Z*,9*Z*)-3,6,9-nonadecatriene
3	1885	*n*-hexadecanol (*present in very few females*)
4	1900	*n*-nonadecane
5	1960	palmitic acid
6	2007	*n*-hexadecyl acetate (*present in very few females*)
7	2024	*n*-octadecanal
8	2074	(3*Z*,6*Z*,9*Z*)-3,6,9-henicosatriene
9	2100	*n*-henicosane
10	2127	methyl stearate (internal standard)
11	2200	*n*-docosane
12	2229	*n*-icosanal
13	2275	*n*-icosanol
14	2300	*n*-tricosane
15	2400	*n*-tetracosane
16	2500	*n*-pentacosane
17	2573	3-methylpentacosane
18	2600	*n*-hexacosane
19	2662	2-methylhexacosane
20	2700	*n*-heptacosane
21	2800	*n*-octacosane

[a] Numbers correspond to peaks in chromatogram in Fig. 1

9.7:1), which was significantly different from the ratio found for moths from Tasmania (*t-test*, t=7.614, df=26, P<0.001). Dissections revealed that all of these females had mated once.

SIM analysis of solvent extracts from the abdominal tips of nine male *M. privata* from Tasmania, detected C21 triene in six individuals, and (3*Z*,6*Z*,9*Z*)-3,6,9-tricosatriene (C23 triene) was tentatively identified in all individuals. No C19 triene was found in any of the male extracts. Levels of both trienes varied widely between males as did the ratio of C23: C21 triene. Mean estimated of C21 triene titers in extracts of males was 1.56 ng (± 0.3 SE, range=415 pg to 2.27 ng), which was not significantly different from that found in female extracts (*t-test*, t=0.24, df=19, P>0.05). Mean estimated titers of C23 triene was 7.33 ng (± 1.7 SE, range 961 pg to 16.71 ng), while the mean ratio of C23:C21 triene was 5.2:1 (± 1.1 SE, range 2.3:1 to 10.6:1).

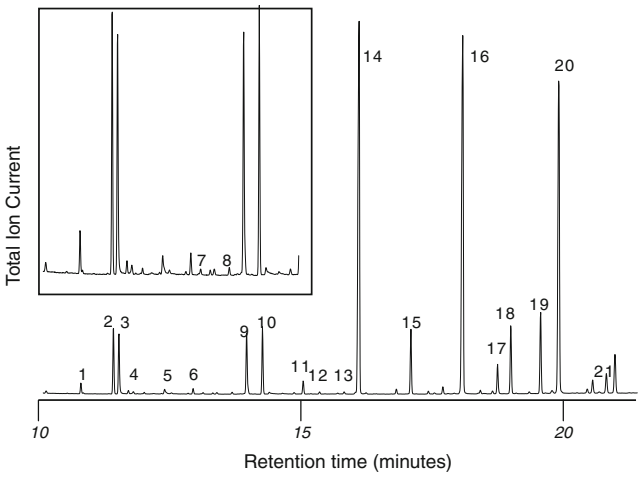


Fig. 1 Representative total ion current chromatogram of extract from a female *Mnesampela privata* abdominal tip. Number designations for peaks are as listed in Table 1. Insert shows magnified portion of chromatogram to indicate where compounds 7 (*n*-octadecanal) and 8 ((3*Z*,6*Z*,9*Z*)-3,6,9-henicosatriene), eluted

EAG and GC-EAD EAG responses of male *M. privata* antennae to C19 and C21 trienes differed (Fig. 4). Antennal responses to 5 μg of C21 triene were consistently higher than the blank stimulus, but lower than that to 5 μg C19 triene (P<0.05, LSD). EAG response to C21 triene was greater than that of 1-octadecanol and hexadecyl acetate (P<0.05, LSD), which elicited an antennal response similar to the blank stimulus. When C21 triene was added to 5 μg C19 triene at a range of ratios, including similar to that found in female extracts, there was a slightly but not significantly enhanced antennal response (P>0.05, LSD) (Fig. 4).

GC-EAD runs that used the combined abdominal extracts from three female *M. privata*, which had relatively high levels of C19 and C21 trienes as previously determined by GC-MS, elicited a weak responses (<2 mV) in antennae of males to the C19 triene. No antennal response or FID peak was detected in the region where the C21 triene was expected to elute.

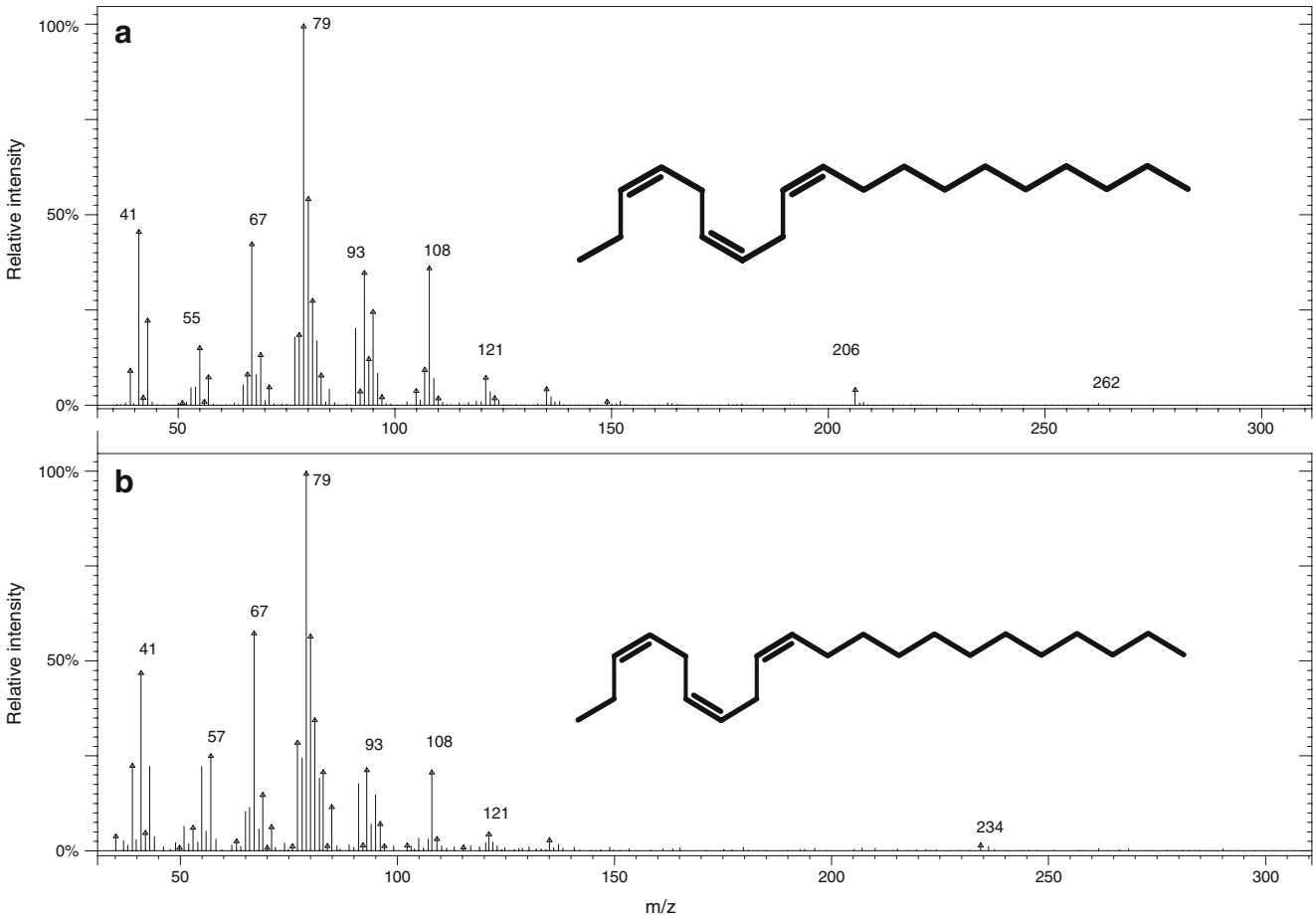


Fig. 2 EI mass spectra of C19 (**a**) and C21 (**b**) trienes from female *Mnesampela privata*

Field Trials

Experiment 1. Field Bioactivity of C19 and C21 Trienes: Significantly more *M. privata* males were caught in traps baited with a 40:1 ratio of C19:C21 trienes than in traps baited with C19 triene alone, but there was no significant difference in the number of males caught in traps baited with 1 mg, 5 mg or 10 mg C19 triene (ANOVA, $\log_{10} + 1$ transformed counts, F=4.9, df=62, P<0.05, followed by LSD test at P=0.05) (Table 2). The majority of males were caught at Surrey Hills, NW Tasmania, where a high *M. privata* population developed in late summer 2007. No *M. privata* were caught in traps placed in plantations in Victoria/South Australia, and trap captures were also low in Western Australia where trials were conducted late in the flight season. Across all plantations (N=18 locations), *M. privata* males were caught at 61.1% of locations when traps were baited with a 40:1 blend of C19:C21 trienes compared to 16.7% when traps were baited with 1 mg C19 triene only.

A large number of *Androchela smithi* McQuillan (Ennominae) also were caught in traps at Surrey Hills, north western Tasmania, during the *M. privata* flight period; 194 in 1 mg C19 triene only baited traps, and 60 in traps baited with a 40:1 ratio of C19:C21 triene. At this location, three moths of an unidentified geometrid species were also caught in C19 triene traps in March 2008. In traps placed in southern Tasmania, 12 *Mnesampela heliochrysa* (Lower) were caught in C19 triene only baited traps, but none were caught in 40:1 ratio of C19:C21 triene baited traps

Experiment 2. Optimal Ratio of C19 and C21 Trienes for Trapping M. privata: A total of 868 male *M. privata* were caught in five replicate traps of eight test baits between 19 December 2007 and 18 March 2008, at Surrey Hills, NW Tasmania. Lures containing C19 and C21 trienes in a ratio of 16:1, 33:1, and 40:1 caught significantly more *M. privata* males than those with a 3:2 and 67:1 ratio or C19 triene only (Table 3). Traps baited with C21 triene only (loaded at an amount equivalent to that in the 40:1 ratio C19:C21 triene baits) or hexane only did not catch any male *M. privata*. When trap catch was expressed as the proportion of male *M. privata* caught in each bait type at each replicate site, captures in 16:1 ratio of C19:C21 trienes were significantly higher than other blends (Fig. 5).

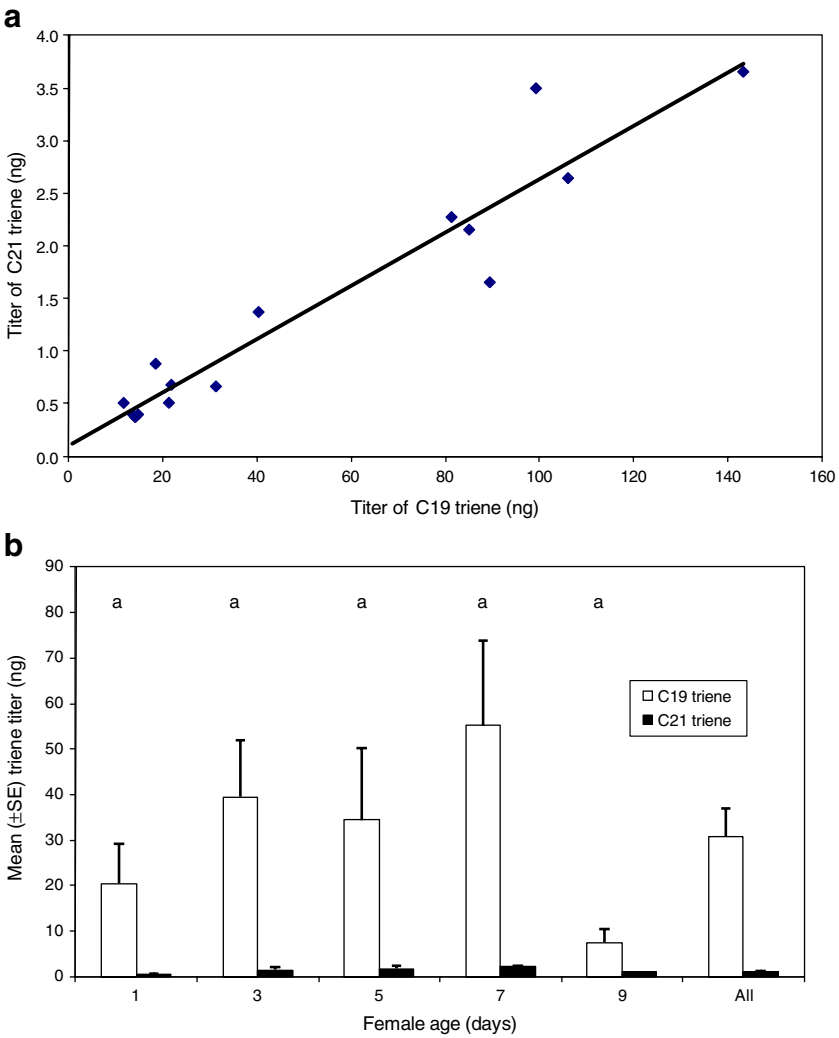


Fig. 3 **a** Correlation between estimated titers of C19 and C21 trienes in individual, unmated, female *Mnesampela privata* abdominal tip extracts, originating from Tasmania (N=15), calculated as methyl stearate equivalents (see text). Levels were positively correlated (Pearson's correlation coefficient=0.956, P<0.001). **b** Mean (± SE) estimated titers of C19 and C21 trienes (nanograms) in abdominal tip extracts of unmated female *M. privata*, originating from Tasmania 1, 3, 5, 7 and 9-d-old and for all moths analysed (C19 triene: N=10, 7, 10, 5, 5 and 37 females, respectively; C21 triene: N=2, 4, 5, 3, 1, and 15 females, respectively). Letters above C19 triene means indicate that they were not significantly different by ANOVA (F=1.4, df=36, P=0.256). C21 triene means were not statistically compared due to the small sample size in some age categories

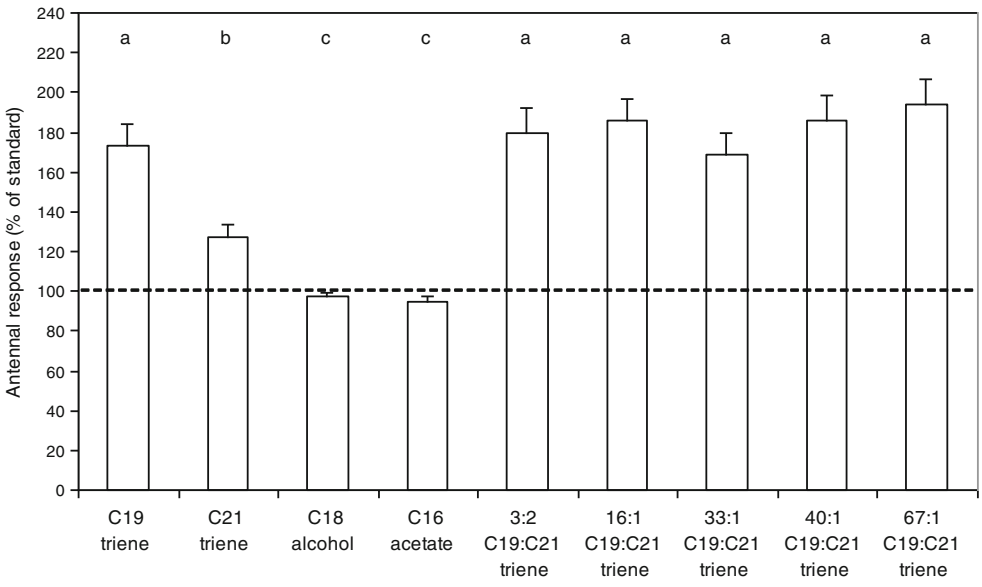


Fig. 4 Mean (± SE) EAG response of male *Mnesampela privata* antennae to 5 μg of C19 triene, C21 triene, 1-octadecanol (C18 alcohol), hexadecyl acetate (C16 acetate) and various blends of C19:C21 trienes dissolved in hexane, expressed as a percentage of the control stimulus (dashed line). Means with different letters atop bars are significantly different by one-way ANOVA (F=12.18, df=75, P<0.001) followed by the LSD test (P=0.05). Number of antennae tested: C19 and C21 trienes alone N=12, C18 alcohol and C16 acetate N=4 and triene blends N=8

Table 2 Field captures of *Mnesampela privata* in traps baited with C19 triene alone or C19 triene + C21 triene (40:1 ratio) placed in *Eucalyptus* plantations at various locations around Australia

Lure composition		Total number of males captured/location[a]				
C19 triene (mg)	C21 triene (μg)	NW Tasmania	NE Tasmania	Southern Tasmania	Victoria/South Australia	Western Australia
1	0	45	0	0	0	0
5	0	21	0	0	NT[b]	NT
10	0	22	0	0	0	1
1	25	456	25	21	0	12

[a] Traps replicated three times each location except Victoria/South Australia and Southern Tasmania where four and five replicates were employed, respectively. Trapping was conducted in Tasmania April 2007–February 2008, Victoria/South Australia March–May 2007 and Western Australia April–June 2007

[b] *NT* not tested

A total of 73 *A. smithi* were caught in traps baited with C19 triene only (mean=14.6 moths/trap) compared to 2, 5, 2, 3, and 2 *A. smithi* caught in 3:2, 16:1, 33:1, 40:1, and 67:1 ratio of C19:C21 triene, respectively (overall mean for blends=0.6 moths/trap). While no *M. privata* were caught in traps baited with C21 triene only, 1 *A. smithi* (mean=0.6 moths/trap) and 1 unidentified geometrid were caught. One unidentified species of geometrid also was caught in a trap baited with a 3:2 ratio of C19:C21 triene. No moths were caught in the control traps.

Experiment 3. Comparison of Trap Design: The mean number of male *M. privata* caught in Delta traps (33.0± 1.4 SE, range 29–36 moths per trap) was higher than in Unitraps (11.6±2.5 SE, range 7–19 moths per trap) (*t*-test, $t=7.31$, $df=8$, $P<0.001$).

Experiment 4. Effect of Lure Age on Trap Catch: There was no significant difference in the number of male *M. privata* caught in traps baited with new lures of 16:1 C19:C21 trienes (mean=19.6±1.2 SE) vs. lures aged in the field for 90 days (mean=23.4±2.1 SE) (*t*-test, $t=1.12$, $df=8$, $P>0.05$).

Discussion

We identified (3*Z*,6*Z*,9*Z*)-3,6,9- henicosatriene as a second bioactive compound in the sex pheromone of *M. privata*, in addition to the major component (3*Z*,6*Z*,9*Z*)-3,6,9-nonadecatriene. Field trials in Tasmania demonstrated that the addition of 1–6% of C21 triene to 1 mg C19 triene significantly increased the number of male *M. privata* caught in traps. Addition of C21 triene to C19 triene also increased the sensitivity of pheromone traps to detect low populations of *M. privata* in *Eucalyptus* plantations, and significantly reduced cross-attraction of other geometrid species. Essential minor pheromone components have been documented in several other geometrid species that utilize polyene hydrocarbons. For example, Szöcs et al. (2004) found that captures of male *Operophtera fagata* Scharf. increased significantly when as little as 0.1% of (1,3*Z*,6*Z*,9*Z*)-nonadecatetraene was added to the main component, (6*Z*,9*Z*)-nonadecadiene.

C19 and C21 trienes are components of the female sex pheromones in many species of Geometridae, Noctuidae, Arctiidae, and Lymantriidae (El-Sayed 2008). To our knowledge, identification of both C19 and C21 trienes in the same sex pheromone has been verified in only one other

Table 3 Field captures of M*nesampela privata* in traps baited with various blends of C19 triene and C21 triene

Traps replicated five times and placed in *E. nitens* plantations at Surrey Hills estate, NW Tasmania, from December 19, 2007 to March 18, 2008

[a] Captures followed by different letters are significantly different by ANOVA ($\log_{10}$ + 1 transformed counts), $F=20.75$, $df=39$, $P<0.001$, followed by the LDS test ($P=0.05$)

Lure composition (μg)			Total number of male *M. privata* caught[a]
C19 triene	C21 triene	~ ratio C19:C21	
1000	0	C19 only	50 a
0	25	C21 only	0 b
1000	15	67:1	63 a
1000	25	40:1	185 c
1000	30	33:1	189 c
1000	60	16:1	324 c
1000	660	3:2	21 a
0	0	control	0 b

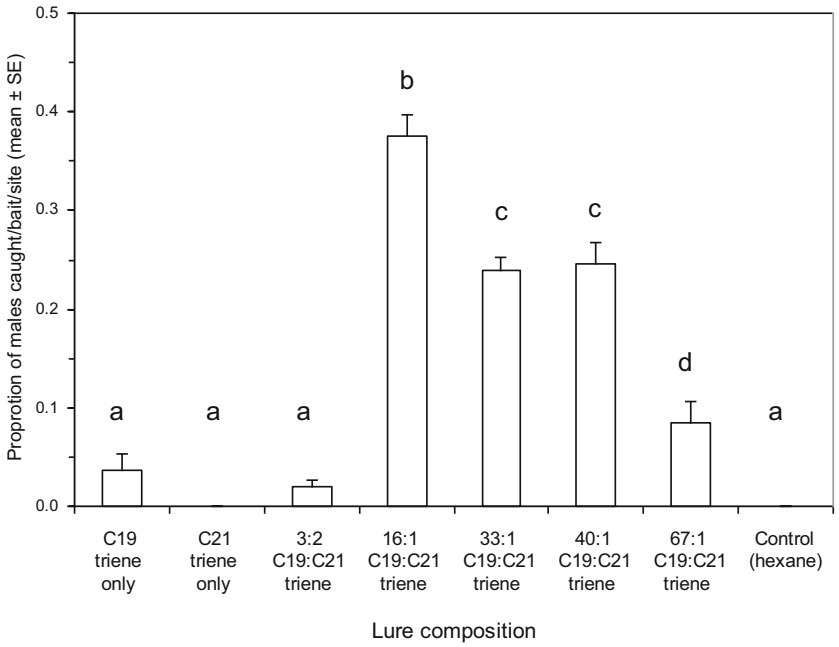


Fig. 5 Mean proportion (± SE) of *Mnesampela privata* males caught at each site (N=5) in Unitraps baited with 1 mg C19 triene alone, 0.25 µg C21 triene alone, hexane only or 1 mg C19 triene with varying amounts of C21 triene to give indicated ratios. Means with different letters atop bars are significantly different by ANOVA (F=82.54, df=39, P<0.001) followed by the LSD test (P=0.05). Trapping was conducted between 19 December 2007 and 18 March 2008 in *E. nitens* plantations at the Surrey Hills estate, NW Tasmania

species, *Colotois pennaria* L. (Szöcs et al. 1993; El-Sayed 2008), which belongs to the same subfamily as *M. privata*. Millar et al. (1992) identified both C19 and C21 trienes in pheromone gland extracts of *Epirrhoe sperryi* (H.). However, only C19 triene was attractive in the field, despite EAG activity of C21 triene in the laboratory. Wong et al. (1985) and Millar et al. (1991) reported that males of several other species of Geometridae and Noctuidae responded to both C19 and C21 trienes during electroantennogram testing, but the actual presence of these compounds in the sex pheromone was not determined, nor was the attraction to both trienes tested in the field. Several species of moths utilize blends of polyene hydrocarbons with mixed chain lengths, but usually the blend consists of compounds differing by only one carbon (Millar 2000). Therefore, *M. privata* and *C. pennaria* appear to be unique in that they use a blend of 3*Z*,6*Z*,9*Z* trienes that differ by two carbons.

While levels of the C19 and C21 trienes in individual females varied widely, the ratio of these two compounds was more constant and highly correlated (Fig. 3a). In Tasmanian *M. privata*, the mean ratio of C19:C21 trienes changed linearly from a 29:1 in 1-d-old females to 43:1 in 7-d-old females. Although this difference was not significant here, Allison and Cardé (2006) found that the ratio of pheromone components in a pyralid moth (*Cadra cautella* (Walker)) changed with female age, and suggested that males could use this to assess the reproductive value of females, which declined markedly with age. If this is the case in *M. privata*, it may explain why significantly more males were caught in traps baited with a lower ratio of C19:C21 triene (16:1) than in traps baited with a ratio closer to the average found in females (33:1) (see also: Szöcs et al. 1993; Strong et al. 2008).

GC-MS analysis of gland extracts from females caught in a light trap near Albany, Western Australia, found significant differences in the ratio of the two trienes compared to populations from Tasmania. Preliminary field trials in Western Australia suggested that increased trap sensitivity could be gained from the addition of more C21 triene to C19 triene but further research is needed to confirm the optimal ratio of these compounds to use for monitoring male *M. privata* in this and other geographical areas.

In their trapping study that used C19 triene baits, Steinbauer et al. (2004) reported catching three closely related species of geometrid: *Dolabrossa amblopa* Guest, *Mnesampela heliochrysa*, and *M. arida* McQuillan. In our study, we caught large numbers of *A. smithi* and *M. heliochrysa* in Tasmania during the flight period of *M. privata*. No *M. heliochrysa* were caught in traps baited with a blend of C19 and C21 trienes thus suggesting that the latter compound may act as a behavioral antagonist to this species. Little is known about the biology of *A. smithi*, which was described relatively recently and has been previously collected only from the Tasmanian highlands (McQuillan 1996). Captures of *A. smithi* occurred in traps baited with a range of blends of C19 and C21 trienes, but were significantly lower than in traps baited with C19 triene alone. Similarly, in field trials in Canada, Millar et al. (1992) reported the indiscriminate cross attraction of *E. sperryi* to lures containing C19 triene regardless of whatever other components were added, suggesting that other physiological or behavioral

mechanisms (e.g., closely synchronized female calling and male response) minimize male attraction to pheromone blends produced by female moths of sympatric species.

Our discovery that male *M. privata* also produce the C21 triene found in females is similar to the finding of Heath et al. (1988). They also found that abdominal tip extracts from males of the noctuid *Anticarsa gemmatalis* (Hübner) contained C21 triene, which is a major component of the female sex pheromone of this species, and suggested that it is a hairpencil compound used during courtship.

In summary, we recommend that traps used to monitor *M. privata* populations in Tasmania be baited with lures that contain both C19 and C21 trienes, at a ratio of 16:1. However, further research is needed to verify whether this is an optimal ratio of trienes to use for monitoring *M. privata* in other geographical areas. We also recommend the Delta trap design for monitoring *M. privata* in preference to the Unitrap design due to lower costs and more efficient capture rates. In the light of the recommendation to include C21 triene in the pheromone bait, the relationship between trap catch and subsequent *M. privata* egg and larval infestations needs to be re-examined as Östrand's et al. (2007) trapping studies were conducted with baits containing C19 triene only. The use of synthetic pheromone for the control of *M. privata*, through mating disruption or mass trapping, is not economically feasible at present given the high costs associated with the synthesis of the C19 and C21 trienes and the sporadic nature of this pest.

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were analyzed initially by gas chromatography mass spectrometry to confirm the presence and Kovat's Indices of 25 cuticular hydrocarbons (CHs) identified by Rivault et al (1998). The amount of each of these CHs in extracts was determined using a Varian CP3380 gas chromatograph, fitted with a flame ionization detector, a CP-sil/5CB column (Varian Inc., 25 m×0.32 mm ID) and a split–splitless injector (split-flow=60 ml min^{-1}; opening 30 sec after injection) with helium as carrier gas (50 cm sec^{-1}). The oven was programmed from 140°C (2 min.) to 280°C at 5°C min^{-1}. CH proportions in control and spinosad-treated individuals (N=10) were compared using a t-test after arcsine transformation to normalize the data.

Statistical analyses were carried out using XLSTAT 2007 software (Addinsoft, New York, NY)

Results

The attractiveness of control female and male extracts was significantly different (Fig. 1; Chi_{15}^2=82.2; P<0.001); control female extract attracted significantly more control insects (96.7% males and 100% females) than did control male extract (90% males and 80% females).

Spinosad treatment produced several behavioral effects: (1) extract of treated females was less attractive than extract of untreated females to both control male and female cockroaches; there was no difference between attractiveness of extracts of treated and untreated males to control insects (Fig. 1a); (2) the responses of treated males to all extracts was low, although their response to control female extract was slightly higher (40%) than to all the other extracts (each 30%); (3) treated females responded similarly to extracts of males and females, whether they were from controls (50–60% for males and females) or treated (30% each from males and females) insects.

Spinosad treatment gave a significant decrease (10–30%) in the relative amounts of n-octacosane, 4-methyloctacosane, and n-nonacosane in both sexes, of 5-methylnonacosane and 3-methylnonacosane in males, and of 5,9- & 5,11-dimethylhentriacontane in females (Table 1).

Discussion

B. germanica males and females both were attracted more to female than to male cuticular extract. This confirms previous work by Tsai and Lee (1997), that showed a preference by males for female stimuli, but it contradicts their observed lack of female preference to female or male stimuli.

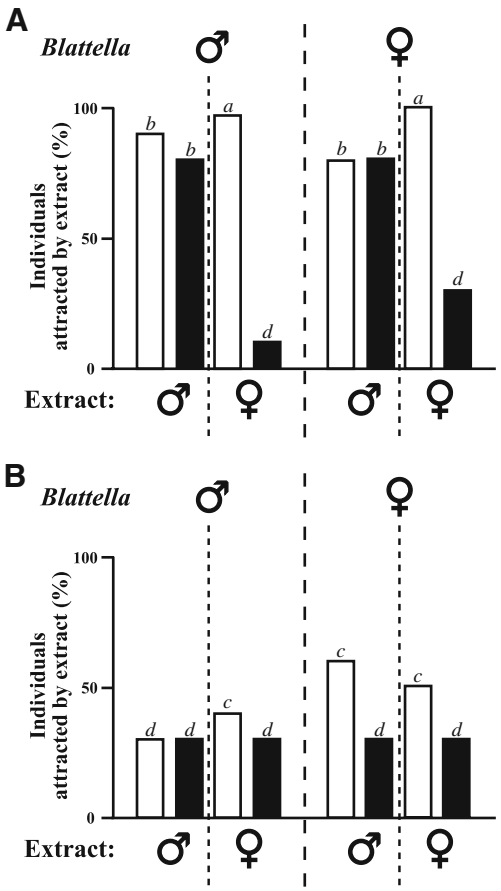

Fig. 1 Responses of control (**a**) and spinosad-treated (**b**) adult male and female *Blatella germanica* to hexane extracts of untreated (*open bars*) and spinosad-treated (*filled bars*) males and females. Cockroaches were tested in a Y-maze olfactometer. The frequencies of attracted insects were analyzed using a Chi-square test; identical *lowercase letters in italics* indicate values that were not significantly different from each other; N=10–30

We observed two effects of spinosad treatment on *B. germanica* chemical communication. First, responses of treated (male and female) adults to male or female control extracts were lower than for controls. Second, the attractiveness of extract from treated females to control adults (both male and female) was lower than that for extract of untreated females. As spinosad acts on the insect nervous system (Watson 2001), its effect on cockroach responses is not surprising. However, its effect on the attractiveness of female extracts was unexpected.

In *B. germanica*, male courtship is modulated by two pheromones: a female-produced contact pheromone (Eliyahu et al. 2008), and a female-produced volatile pheromone, blattellaquinone, which attracts males over a distance (Nojima et al. 2005). Spinosad affected quantities of four (CHs 7, 12, 15, and 17; see Table 1) of the six CHs that are described as aggregation cues in *B. germanica* (Rivault et al. 1998), which could, in turn, influence

Table 1 Proportion of the cuticular hydrocarbons (CHs) of control and spinosad-treated male and female *Blattella germanica*. In each sex, the proportion of each compound was compared between control and treated cockroaches by a bilateral *t*-test (after an *Arcsin* transformation)

CH[a]		Control males	Treated males	t_{18df}	*P*	Control females	Treated females	t_{18df}	*P*
1	*n*-Heptacosane	3.72± 0.54	2.97±0.54	ns		4.27±0.46	3.15±0.54	ns	
2	9-,11- & 13-Methylheptacosane	6.88±0.57	8.32± 0.92	ns		5.12± 0.36	4.52± 0.68	ns	
3	5-Methylheptacosane	4.12 ± 0.27	4.42± 0.51	ns		3.76±0.15	3.36±0.49	ns	
4	11,15-Dimethyheptacosane	0.70±0.06	0.82± 0.08	ns		0.96±0.11	0.89±0.11	ns	
5	3-Methylheptacosane	4.29±0.14	4.25±0.45	ns		5.23±0.44	4.40±0.43	ns	
6	5,9- & 5,11-Dimethyheptacosane	1.33±0.22	1.19±0.09	ns		1.36±0.09	2.16±0.55	ns	
7	*n*-Octacosane	1.02± 0.09	0.71± 0.07	2.83	0.011	1.16±0.09	0.90±0.05	2.51	0.022
8	3,11- & 3,9-Dimethyheptacosane	1.36±0.14	1.38±0.09	ns		1.77±0.14	3.03±0.82	ns	
9	12- & 14-Methyloctacosane	1.53±0.06	1.52± 0.07	ns		1.41± 0.04	1.42± 0.09	ns	
10	2-Methyloctacosane	1.00±0.02	0.98±0.03	ns		1.36±0.05	0.88±0.27	ns	
11	4-Methyloctacosane	0.72± 0.01	0.66±0.02	2.84	0.011	0.95±0.03	0.85±0.02	2.69	0.015
12	*n*-Nonacosane	7.48±0.50	5.15±0.10	3.20	0.005	7.66±0.52	6.54±0.27	2.10	0.050
13	9-,11-, 13- & 15-Methylnonacosane	20.36±0.54	20.55±0.91	ns		13.79±0.30	14.33±0.29	ns	
14	7-Methylnonacosane	2.82± 0.13	2.98±0.13	ns		2.41± 0.13	2.58±0.12	ns	
15	5-Methylnonacosane	7.11± 0.29	6.40±0.18	2.11	0.049	5.93±0.31	5.84±0.23	ns	
16	11,15- & 13,17-Dimethylnonacosane	4.77±0.40	5.72± 0.40	ns		6.76±0.52	7.11± 0.51	ns	
17	3-Methylnonacosane	9.50±0.61	7.31± 0.21	3.30	0.004	9.85±0.32	9.22± 0.52	ns	
18	5,9- & 5,11-Dimethylnonacosane	4.21± 0.30	4.76±0.22	ns		4.70±0.21	4.79±0.47	ns	
19	3,7-, 3,9- & 3,11-Dimethylnonacosane	12.78± 1.04	14.68±0.99	ns		16.55± 1.33	19.17±0.77	ns	
20	11-, 13- & 15-Methyltriacontane	1.91± 0.22	2.19±0.19	ns		2.42± 0.17	2.41± 0.17	ns	
21	4,8- & 4,10-Dimethyltriacontane	0.40±0.04	0.76±0.30	ns		0.67±0.05	0.62± 0.05	ns	
22	11-, 13- & 15- Methylhentriacontane	1.22± 0.11	1.27±0.31	ns		0.66±0.04	0.77±0.05	ns	
23	13,17- & 11,15-Dimethylhentriacontane	0.25±0.03	0.38±0.07	ns		0.37±0.02	0.42± 0.04	ns	
24	5,9- & 5,11-Dimethylhentriacontane	0.36±0.06	0.42± 0.06	ns		0.71± 0.09	0.48±0.07	2.17	0.044
25	10 & 12-Methyldotriacontane	0.17±0.03	0.22± 0.04	ns		0.15±0.02	0.17±0.01	ns	

t-Value (18 df) and the corresponding *P* are provided when significant.

[a] The CHs are identifed following Rivault et al. (1998).

aggregation responses to treated cockroaches. However, this would not explain why we obtained reduced attraction to treated cockroach extract in the olfactometer. Future work should investigate whether spinosad treatment affects blattellaquinone production.

We have shown previously that other insecticides affect the CH profile in *B. germanica*. A sub-lethal dose of boric acid feminized the CH profile of males but did not change female the CH profile (Kilani-Morakchi et al. 2005). Halofenozide (an ecdysteroid agonist) decreased CH levels, probably by affecting synthesis and/or transport (Kilani-Morakchi et al. in press). Work is needed to determine precisely how these chemicals influence CH production in insects.

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glucose moiety and the formation of deterrent breakdown products, including nitriles, isothiocyanates, thiocyanates, oxazolidine-2-thiones, and epithionitriles (Grubb and Abel 2006; Halkier and Gershenzon 2006). More than 120 different glucosinolate side chains have been identified (Fahey et al. 2001), and at least 37 of these are found in different isolates of the well-studied model plant *Arabidopsis thaliana* (Reichelt et al. 2002).

Plutella xylostella, as a crucifer specialist, has evolved an effective detoxification system through the use of sulfatases that change the glucosinolate chemical structure to prevent the action of myrosinase enzymes on this substrate. Consequently, fewer toxic glucosinolate breakdown products are formed (Ratzka et al. 2002). With this ability to freely exploit cruciferous plants as a food source, *P. xylostella* actually uses components of the glucosinolate-myrosinase system as highly predictable oviposition cues. Glucosinolate-containing fractions of extracts of the cruciferous crop species *Brassica napus, B. juncea*, and *Sinapis alba* were found previously to attract ovipositing *P. xylostella*. These extracts contain a mixture of glucosinolates, including those with indole side chains. They were found to stimulate oviposition, but lost their activity upon *in vitro* hydrolysis by myrosinase, indicating that intact glucosinolates can function as oviposition cues for *P. xylostella* (Reed et al. 1989). However, specific fractions of glucosinolate breakdown products from cabbage leaves have been found to significantly increase oviposition by *P. xylostella* (Hughes et al. 1997). These active compounds were identified later as the aliphatic glucosinolate-derived breakdown products iberin and sulforaphane, both of which are isothiocyanates that stimulate *P. xylostella* through olfaction (Renwick et al. 2006). Whereas the *in vitro* role of aliphatic glucosinolate breakdown products and intact indole glucosinolates on oviposition by *P. xylostella* is documented, the attractive effects of these compounds have not been studied well *in vivo*.

A multitude of mutant and transgenic isolates of *A. thaliana* provide an excellent foundation for the study of the chemical attractants involved in *P. xylostella* oviposition. *Arabidopsis thaliana* with T-DNA knockout mutations of both CYP79B2 (At4g39950) and CYP79B3 (At2g22330), two cytochrome P450s that lead to the production of indole-3-acetaldoxime, are almost completely blocked in the production of indole glucosinolates (Zhao et al. 2002). The transcription factors MYB28 (At5g61420) and MYB29 (At5g07690) previously have been identified as regulators of methionine-derived glucosinolates biosynthesis (Gigolashvili et al. 2007, 2008), and the *myb28 myb29* double mutant is almost completely devoid of aliphatic glucosinolates (Sønderby et al. 2007; Beekwilder et al. 2008). The two double mutants make it possible to study the *in planta* function of these two major classes of glucosinolates, aliphatic and indole, on *P. xylostella* oviposition. Furthermore, the inactivation of TGG1 (At5g2600) and TGG2 (At5g25980), the two predominant above-ground myrosinases of *A. thaliana* largely eliminates damage-induced glucosinolate breakdown (Barth and Jander 2006). The *tgg1 tgg2* double mutant allows examination of the effect of glucosinolate breakdown on oviposition by *P. xylostella*. To determine which components of the glucosinolate-myrosinase system serve as signals for ovipositing *P. xylostella*, we performed bioassays on extracts and intact plants impaired in different aspects of this chemical defense. We demonstrated that intact indole glucosinolates and hydrolysis products of aliphatic glucosinolates function as important oviposition stimuli *in vivo*. In particular, an indole glucosinolate with secondary modifications is used as a reliable host recognition cue by *P. xylostella* females.

Methods and Materials

Plants and Insects Wildtype *A. thaliana* land race Columbia-0 (Col-0) seeds were obtained from the Arabidopsis Biological Resource Center (ABRC, www.arabidopsis.org). The Col-0 *cyp79B2 cyp79B3* (Zhao et al. 2002) and *cyp81F2* (Clay et al. 2009) mutants were kindly supplied by J. Celenza (Boston University, Boston, MA, USA) and N. Clay (Massachusetts General Hospital, Boston, MA, USA), respectively. Creation of the *tgg1-3 tgg2-1* and *myb28-1 myb29-1* double mutants by crossing the T-DNA insertion lines SAIL_786_B08 (*tgg1-3*) and SALK_038730 (*tgg2-1*), and SALK_136312 (*myb28-1*) and GABI_868E02 (*myb29-1*), respectively, has been described previously (Barth and Jander 2006; Sønderby et al. 2007). The quadruple mutant *cyp79B2 cyp79B3 tgg1 tgg2* was described previously by De Vos et al. (2008). Plants were grown in Conviron (Winnipeg, Canada) growth chambers in 20- x 40-cm nursery flats using Cornell Mix (Landry et al. 1995) with Osmocoat fertilizer (Scotts, Marysville, OH, USA) at 23°C, 60% relative humidity, with a light intensity of 180 $\mu mol^{-2}\ s^{-1}$ photosynthetic photon flux density and a 16:8 h light:dark photoperiod.

Initially, *P. xylostella* eggs were purchased from Benzon Research (Carlisle, PA, USA). Insects were reared to pupation in Petri dishes on an artificial diet (Southland Products, Lake Village, AK, USA) at 23°C. Subsequent generations of *P. xylostella* were reared in the laboratory. Eggs were deposited on aluminum foil dipped in boiled cabbage (*B. oleracea*) extract. Adult moths were placed in lidded paper cups (0.5 l, International Paper, Memphis, TN, USA) that contained aluminum foil to elicit oviposition. After several days, the foil coated in eggs was removed from the cup and placed on the artificial diet.

Generation of the cyp79B2 cyp79B3 myb28 myb29 Quadruple Knockout The *cyp79B2 cyp79B3* double mutant (Zhao et al. 2002) was manually crossed with the *myb28-1 myb29-1* double mutant (Sønderby et al. 2007). F1 plants were self-pollinated, and a total of 384 plants from the segregating F2 population were grown on soil. Theoretically, due to the independent segregation of four genes, we expected to find the quadruple mutant in one out of every 256 plants. Tissue of 3-wk-old *A. thaliana* plants was harvested into liquid nitrogen and desulfoglucosinolates were extracted using the method described previously (Hansen et al. 2007). As a preliminary screen, the 200–300 nm UV absorption spectrum of the desulfoglucosinolate extractions was measured in microplates with a Spectra-Max M5 96 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Thirty samples with low absorption, presumably low in glucosinolates, were identified. Desulfoglucosinolates from these samples were extracted and run on the HPLC according to the method of Hansen et al. (2007). Eight low-glucosinolate samples, candidates for the quadruple mutant, were found. These plants were genotyped by PCR with the previously described primers detecting the wildtype and T-DNA alleles in *MYB28* and *MYB29* (Sønderby et al. 2007). The following gene specific primers for CYP79B2 (primers for *cyp79b2*: 5′-TGGACAAGTATCATGACCCAATCATCCACG-3′ and 5′-AACGGTTTAGCCAGAAACATATCGT-3’) and CYP79B3 (primers *cyp79b3*: 5-‘TGTTCTATGCATG GACTGGTGGTCAACATG-3′ and 5′-AGGAAACCGAT CACTTGACCGCTTG-3′) were used to detect the wildtype alleles. The 5′-primer was used in combination with LBa1 (5′-TGGTTCACGTAGTGGGCCATCG-3′) to verify the presence of the T-DNAs. One plant was homozygous for all four alleles except *MYB28*, another was homozygous for all alleles except *CYP79B2*. The F3 plants were grown in the subsequent generation, and the quadruple mutant was found by PCR analysis. Glucosinolate profiles were confirmed by analyzing F4 seed glucosinolate profiles from ten seeds as described previously (Hansen et al. 2007; Sønderby et al. 2007).

Plant Extraction and Fractionation Whole rosettes of 21 to 24-d-old *A. thaliana* plants were extracted by placing individual rosettes in 5 ml chloroform for 1 h. The solid plant material was then discarded, and the crude plant extract was stored at 4°C. To obtain fractions containing either intact glucosinolates or breakdown products, the chloroform extracts of 6-7 plants were pooled and evaporated to dryness. The extracts, which still contained indole glucosinolates (Supplemental Fig. 1), then were resuspended in acetonitrile for HPLC fractionation. Fractions collected before 15 min in the 35 min run were determined to contain intact glucosinolates (as compared to glucosinolate standards). Fractions collected after 15 min contained glucosinolate breakdown products. The identity of intact glucosinolates was confirmed by comparison to purified glucosinolate standards, including sinigrin as an internal control, using a Waters 2695 HPLC coupled to a Waters 2996 photodiode array detector. For HPLC analysis, the mobile phases were A, water, and B, 90% acetonitrile, at a flow rate of 1.1 ml min^{-1} at 23°C. Column linear gradients for samples were: 0–1 min, 98% A; 1–6 min 94% A; 6–8 min, 92% A; 8–16 min, 77% A; 16–20 min, 60% A; 20–25 min, 0% A; 25–27 min hold 0% A; 27–28 min, 98% A; 28–35 min, 98% A. An analytical HPLC reversed phase column (Lichrosphere 5 RP-18, ECAP 250, 4.6 mm i.d., 5 mm particle size) was used for the analysis.

Purified Glucosinolates and Breakdown Products All intact glucosinolates were purified from cabbage (*B. oleracea*) according to the protocol described by Clay et al. (2009). Indole glucosinolate breakdown products were purchased from Sigma-Aldrich (St. Louis, MO, USA), except for 3,3′-diindolylmethane that was purchased from LKT Laboratories (St. Paul, MN, USA).

Intact indole glucosinolates were dissolved in HPLC-grade water. Oviposition tests to determine relative attractiveness among indol-3-ylmethylglucosinolate (I3M), 1-methoxy-indol-3-ylmethylglucosinolate (1M-I3M), and 4-methoxy-indol-3-ylmethylglucosinolate (4M-I3M) involved 0.5 mM glucosinolate concentrations. A 1 mM glucosinolate concentration was used in tests on the effect of indole glucosinolate breakdown. I3M was treated with myrosinase (Sigma-Aldrich, St. Louis, MO, USA) to catalyze its breakdown. A myrosinase buffer solution was prepared by combining 33 mM sodium phosphate buffer (pH = 7) with 0.3 mM ascorbic acid as a cofactor (Barth and Jander 2006). Tests on commercial glucosinolate breakdown products involved 1 mM solutions in acetonitrile or methanol.

Oviposition Tests on Intact Plants and Detached Leaves For whole plant oviposition assays, 50 adult *P. xylostella* (~50% females) were allowed to oviposit on 14 to 16 plants, paired mutant and wildtype, in complete darkness in a 30×60 cm nursery flat under a dome cover. After 24 h *P. xylostella* eggs were counted. For experiments with damaged plants, each fully expended leaf was damaged across the mid-vein with a forceps.

Experiments with detached leaves were performed in the above-described 0.5 l paper cups. Petioles of wildtype and mutant leaves were cut and mounted in 1% water agar for pair-wise comparisons. Ten moths per cup were allowed to oviposit for 24 h in the dark, and the eggs were counted.

Oviposition Tests on Liquid Solutions Groups of 10 *P. xylostella* pupae (~1:1 sex ratio) were placed into lidded paper cups. These cups were placed in the dark at 23°C for

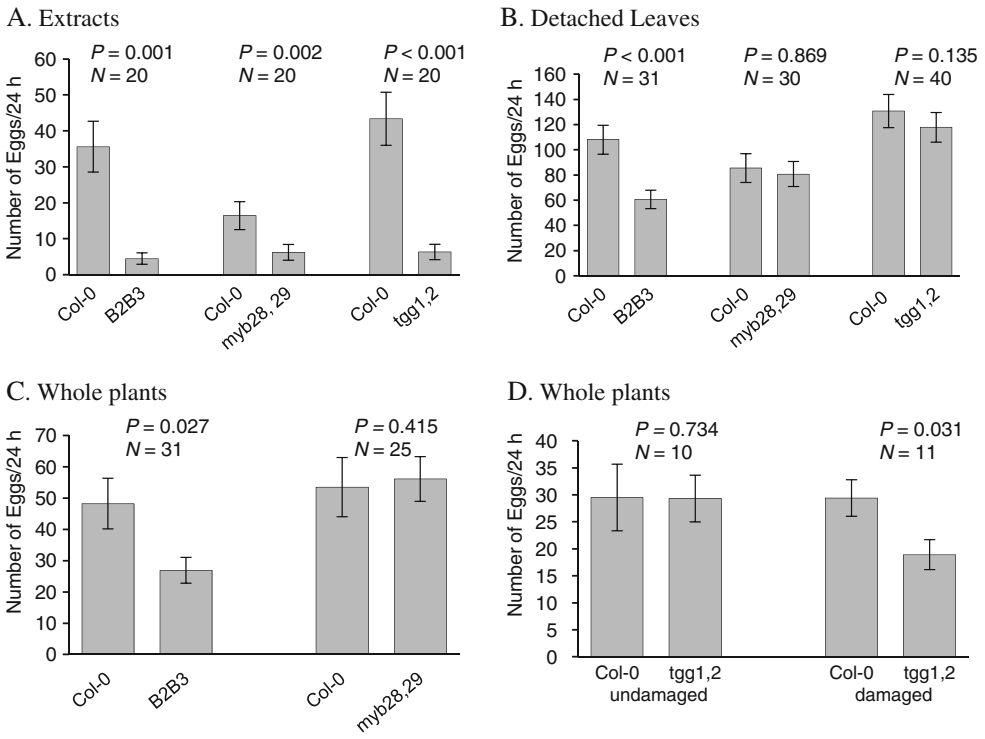

Fig. 1 *Plutella xylostella* 24 h oviposition choice tests comparing the number of eggs laid on Col-0 and double mutants containing low indole glucosinolates (*cyp79B2 cyp79B3*), myrosinase (*tgg1, tgg2*) or aliphatic glucosinolates (*myb28 myb29*), labeled as B2B3, tgg1,2 and myb28,29, respectively. **a** Oviposition tests on crude plant extracts. **b** Oviposition tests on detached leaves. **c** and **d** Oviposition tests on whole plants. (d) Leaves of intact plants were damaged across the mid-vein using a forceps to induce glucosinolate breakdown by myrosinase. Mean ± standard error. (a) and (b) significance determined by Wilcoxon signed rank tests. (c) and (d) significance determined by Mann-Whitney *U*-tests

3–5 d until the majority of the pupae had eclosed. Fifty µl of the leaf extracts were applied to paper towels (C-fold white, Kimberly-Clark, Dallas, TX, USA) and allowed to dry. The extracts were applied within 3.5 cm diam circles marked with a pencil to facilitate egg counting. Each individual paper towel contained two spots with purified glucosinolate-related chemicals in solution or plant extracts to allow for a paired oviposition test. The paper towels then were mounted underneath the lid of the previously described 0.5 l paper cups that contained 10 moths. The moths in the cups were allowed to oviposit for 24 h in the dark. After this period, the cups were placed into a cold room (4°C) to stop oviposition, and the *P. xylostella* eggs within the spots on the paper towels were counted.

Glucosinolate Analysis Glucosinolate extraction and analysis was performed on whole rosettes of 24-d-old plants. Samples were freeze-dried, weighed, and glucosinolates were extracted and analyzed according to the method described by Barth and Jander (2006). Glucosinolate concentrations are expressed as µmol per gram dry weight.

Statistical Analyses Data from oviposition tests done in cups on detached leaves, leaf extracts, or intact indole glucosinolates were analyzed by Wilcoxon signed rank tests. Data from oviposition tests on whole plants and breakdown products were analyzed by Mann-Whitney *U*-tests. All statistical analyses were performed using JMP 2007 for Windows (SAS Institute Inc., Cary, NC, USA).

Results

Oviposition of P. xylostella on A. thaliana Mutants with altered Glucosinolate Production We used *A. thaliana* mutants with altered glucosinolate profiles to determine the *in vivo* role of glucosinolates as oviposition attractants. The *A. thaliana* double mutants *cyp79B2 cyp79B3* (low indole glucosinolates), *myb28 myb29* (residual aliphatic glucosinolates) and *tgg1 tgg2* (no glucosinolate breakdown due to mutations in foliar β-glucosidases) (Table S1) offer an extremely useful tool to test the effect of glucosinolates in stimulating *P. xylostella* oviposition. The crude extracts, detached leaves, and wholes plant of *cyp79B2 cyp79B3* all experienced a significant decrease in oviposition by *P. xylostella* compared to Col-0 wildtype plants (Fig. 1a, b, c), indicating that indole glucosinolates serve as important *P. xylostella* attractants *in vivo*. Similarly, we tested the effect of aliphatic glucosinolates. Whereas elimination of aliphatic glucosinolates did not significantly affect oviposition of *P. xylostella* females in experiments with whole plants and detached leaves (Fig 1b, c), crude extracts from *myb28 myb29* received significantly fewer eggs than those from wildtype plants (Fig. 1a), thus suggesting that aliphatic glucosinolate derived breakdown products play a

role in attracting *P. xylostella* oviposition. These data confirm an earlier finding by Renwick et al. (2006) that isothiocyanates from aliphatic glucosinolates stimulate oviposition behavior. Likewise, *tgg1 tgg2* compared against Col-0 displayed no significant difference in attractiveness of either detached leaves or undamaged whole plants (Fig. 1b, d). However, crude extracts made from wildtype and *tgg1 tgg2* plants revealed a preference for wildtype (Fig. 1a). To investigate the importance of glucosinolate breakdown products, we subsequently damaged and compared oviposition on Col-0 (WT) and *tgg1 tgg2* plants. In contrast to results obtained with undamaged plants, we observed significantly more eggs deposited on damaged Col-0 plants (Fig. 1d). Thus, only damaging processes such as extraction and deliberate plant damage produced significantly different oviposition results between the wildtype and *myb28 myb29* or *tgg1 tgg2* plants. In summary, these results indicate that intact indole glucosinolates and aliphatic glucosinolate-derived glucosinolate breakdown products, most likely the previously described isothiocyanates (Renwick et al. 2006), function as oviposition attractants.

In order to investigate whether different classes of glucosinolates have additive affects on *P. xylostella* oviposition, we studied quadruple mutants that lacked multiple components of the glucosinolate-myrosinase system. Under our growth conditions, the *cyp79B2 cyp79B3 tgg1 tgg2* mutant lacks indole glucosinolates and myrosinase activity (Table S1; De Vos et al. 2008). Moreover, we crossed *cyp79B2 cyp79B3* and *myb28 myb29* to create a quadruple mutant that has no detectable production of indole glucosinolates and shows low levels of aliphatic glucosinolates residually (~6% of wildtype, Table S1). In tests using both crude extracts and whole plants, *cyp79B2 cyp79B3 tgg1 tgg2* received significantly fewer eggs than either of the progenitor double mutants (Fig. 2a, b). The same additive effect of decreased oviposition was observed for the c*yp79B2 cyp79B3 myb28 myb29* quadruple mutant, both on crude extracts and on whole plants (Fig. 2c, d). These results are in accordance with our findings that used the double mutants, suggesting that both intact indole glucosinolates and volatile aliphatic glucosinolate-derived isothiocyanates play a role in the attraction of *P. xylostella* females.

Oviposition Assays using Plant Extract Fractions To test the hypothesis that *P. xylostella* is attracted to intact indole glucosinolates for oviposition, the crude extracts of Col-0 and the *cyp79B2 cyp79B3* double mutant were separated by HPLC into two fractions that contained either intact glucosinolates or glucosinolate breakdown products. Wildtype Col-0 fractions containing intact glucosinolates received significantly more eggs than the Col-0 fraction glucosinolate breakdown products (Fig. 3a), suggesting that intact glucosinolates are more attractive to *P. xylostella* females. Moreover, despite having a similar aliphatic glucosinolate profile, the Col-0 fraction that contained intact glucosinolates received significantly more eggs when compared to the same fraction from *cyp79B2 cyp79B3* mutant plants (Fig. 3b; Table S1), indicating that intact indole glucosinolates are an important oviposition cue for *P. xylostella*.

Purified Indole Compounds In vitro tests indicate that I3M alone serves as a strong oviposition attractant (Fig. 4a). The addition of myrosinase to catalyze the breakdown of I3M significantly decreases this attractiveness when compared to intact I3M (Fig. 4b). This result is in agreement with results from the glucosinolate fractionation, thus implicating the importance of intact indole glucosinolates (Fig. 3). Furthermore, tests using six commercially available indole glucosinolate breakdown products indicate that none of these compounds serve as oviposition attractants (Table 1). In fact, indole-3-carbinol was found to have a deterrent effect on oviposition when compared against the solvent-only control. However, mixing indole-3-carbinol with an equivalent amount of the oviposition attractant 4-methoxy-indol-3-ylmethylglucosinolate (4M-I3M) had no effect on oviposition attractiveness compared to 4M-I3M (12.3±2.76 and 10.1± 1.97, respectively; $P = 0.618$; Wilcoxon signed rank test).

Expanding on the research on isolated indole glucosinolates, aqueous solutions of I3M, 4M-I3M, and 1-methoxy-indol-3-ylmethylglucosinolate (1M-I3M) were compared directly against each other in oviposition assays. Female *P. xylostella* moths significantly preferred depositing their eggs on 4M-I3M compared to either of the two other indole glucosinolates (Fig. 4c, d), which were found to be equally attractive (Fig. 4e). Taken together, these results suggest that *P. xylostella* can distinguish secondary modifications in indole glucosinolate chemistry, thus identifying suitable hosts for oviposition.

To investigate the role of 4M-I3M, we made use of a recently described biosynthetic mutant that is disrupted in a cytochrome P450 involved in 4M-I3M production (Bednarek et al. 2009; Clay et al. 2009; Pfalz et al. 2009). Whereas plant extracts from Col-0 received significantly more *P. xylostella* eggs than those from a mutant plant with significantly lower concentrations of 4M-I3M (*cyp81F2*; Fig. 5a), a comparison of wildtype and *cyp81F2* showed no preference for either genotype (Fig. 5b).

Discussion

Our findings demonstrate that intact nonvolatile indole glucosinolates are important *P. xylostella* oviposition

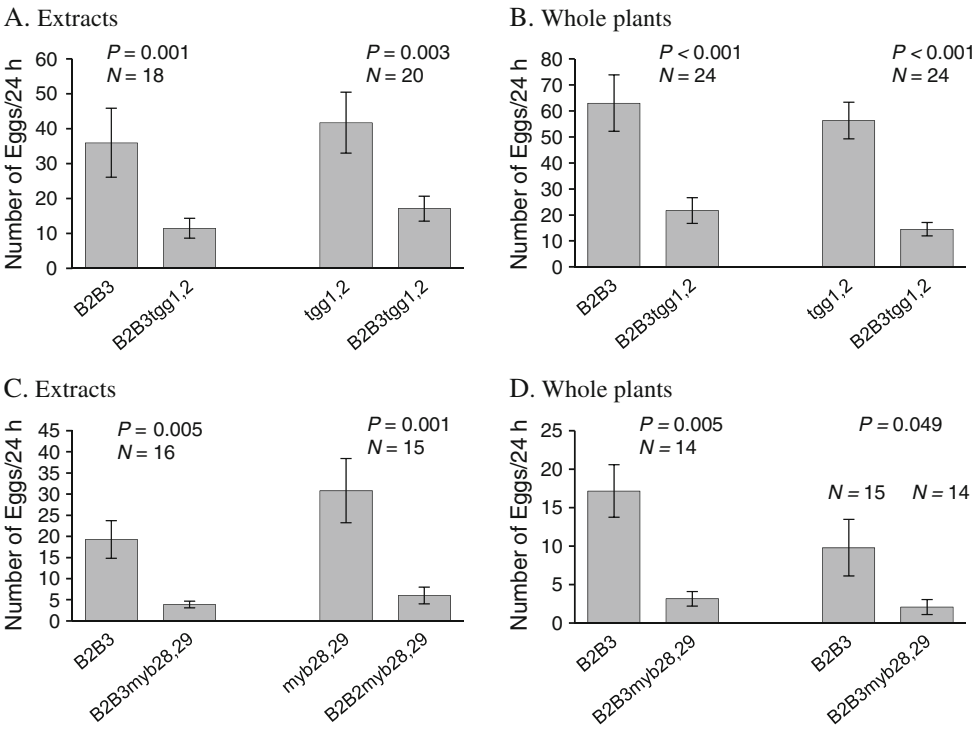

Fig. 2 *Plutella xylostella* 24 h oviposition choice tests comparing the number of eggs laid on previously described double and quadruple mutants with reduced production of either indole glucosinolates and myrosinase (c*yp79B2 cyp79B3 tgg1 tgg2*) or indole and aliphatic glucosinolates (c*yp79B2 cyp79B3 myb28 myb29*). **a**, **b** Oviposition on crude extracts or whole plants of c*yp79B2 cyp79B3 tgg1 tgg2* (B2B3tgg1,2) and its progenitor double mutants. **c**, **d** Oviposition on crude extracts or whole plants of c*yp79B2 cyp79B3 myb28 myb29* (B2B3myb28,29) and its progenitor double mutants. Mean ± standard error. (a) and (c) comparisons for significance determined by Wilcoxon signed rank tests. (b) and (d) significance determined by Mann-Whitney *U*-tests

attractants, and that at least some *P. xylostella* oviposition decisions are made at the leaf surface. Previously implicated as oviposition attractants by Reed et al. (1989), indole glucosinolates now can be linked to *P. xylostella* host plant recognition *in vivo*. Mutant plants disrupted in the biosynthesis of indole glucosinolate exhibited a significant decrease in oviposition when compared to wildtype plants (Fig. 1a, b, c). Extract fractions of these mutant and wildtype plants indicate that intact indole glucosinolates rather than breakdown products serve as the oviposition cues (Fig. 3). Moreover, we demonstrated that myrosinase-catalyzed breakdown eliminates the attractiveness of I3M *in vitro* (Fig. 4b; Reed et al. 1989). This observation also is supported by the failure of several indole glucosinolate breakdown products to elicit any positive oviposition response (Table 1). Altogether, this indicates that *P. xylostella* do not use indole glucosinolate breakdown products for host recognition.

Interestingly, we found that, depending on their secondary modifications, indole glucosinolates have differential attractiveness for *P. xylostella* females. Purified 4M-I3M elicited a significantly higher amount of oviposition than either I3M or 1M-I3M (Fig. 4c, d). Hydrolysis of 4M-I3M by TGG1 and TGG2 myrosinases occurred significantly slower than that of the other indole glucosinolates or aliphatic glucosinolates (Barth and Jander 2006). It is tempting to speculate that *P. xylostella's* preference for this particular indole glucosinolate

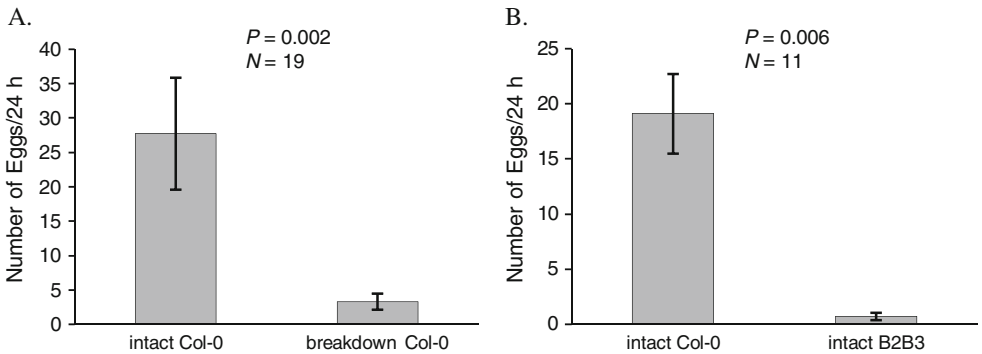

Fig. 3 *Plutella xylostella* oviposition 24 h choice tests comparing the fractionated crude extracts of Col-0 and the low indole glucosinolate expressing double mutant *cyp79B2 cyp79B3* (B2B3). **a** Comparison between the Col-0 fraction containing intact glucosinolates and the Col-0 fraction containing glucosinolate breakdown products **b** Comparison between the Col-0 fraction containing intact glucosinolates and the *cyp79B2 cyp79B3* fraction containing intact glucosinolates. Mean ± standard error, comparisons for significance are Wilcoxon signed rank tests

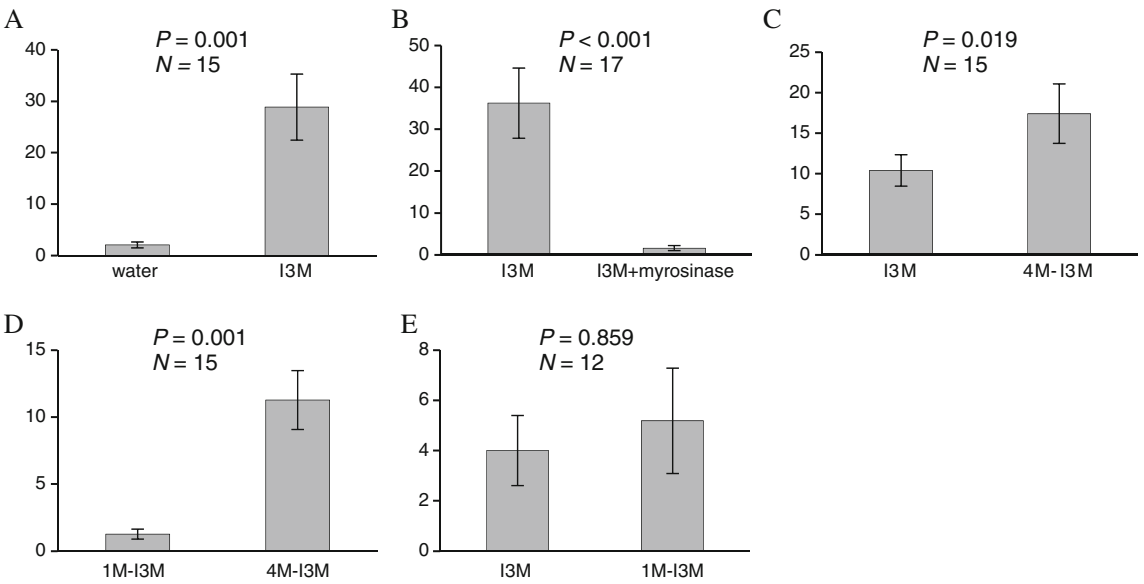

Fig. 4 *Plutella xylostella* 24 h oviposition choice tests comparing the number of eggs laid on purified indole glucosinolates spotted on paper towels. **a** Oviposition on I3M vs. water. **b** I3M and myrosinase buffer vs. I3M alone. **c**, **d**, **e** Comparisons of the three indole glucosinolates, I3M, 4M-I3M, and IM-I3M, against each other. Mean number of eggs laid ± standard error, all comparisons for significance are Wilcoxon signed rank tests

may have arisen due to the increased stability of 4M-I3M, which therefore acts as a reliable host recognition cue. A beneficial role in protecting *A. thaliana* against generalist insect herbivores or pathogenic micro-organisms may explain why the conversion of I3M to 4M-I3M persists in *A. thaliana* and other crucifers. For instance, secondary modifications in indole glucosinolates play an important role in protection against phloem-feeding insects. Kim and Jander (2007) demonstrated that upon infestation with green peach aphids (*Myzus persicae*), *A. thaliana* plants specifically increase conversion of I3M to 4M-I3M, which reduces aphid reproduction *in vitro*. Moreover, mutant plants that lack 4M-I3M biosynthesis, through a mutation in a cytochrome P450 (CYP81F2; At5g57720), were independently shown to have increased *M. persicae* performance in comparison to Col-0 wildtype plants (De Vos and Jander 2009; Pfalz et al. 2009). Here, we showed that the same mutation (*cyp81F2*) leads to reduced oviposition of *P. xylostella* on plant extracts compared to those from wildtype plants (Fig. 5a). In contrast, we did not observe a difference in oviposition between Col-0 and *cyp81F2* in whole plant experiments (Fig. 5b). Perhaps the significant increases in intact I3M levels in *cyp81F2* plants (Bednarek et al. 2009; Clay et al. 2009) are sufficient to mask the lack of 4M-I3M. Alternatively, it has been demonstrated that indole glucosinolate distribution in leaves is not homogenous. In line with the anti-aphid properties of 4M-I3M, this indole glucosinolate could be phloem-specific and not located at outer lamina where other intact indole glucosinolates have been found (Shroff et al. 2008).

Aliphatic glucosinolate breakdown products, which previously have been demonstrated to attract *P. xylostella* oviposition through olfactory stimulation (Renwick et al. 2006), are most likely the factors that cause the differences in oviposition preference observed in our tests on both aliphatic glucosinolate and myrosinase-deficient mutants compared to the wildtype. The *myb28 myb29* and *tgg1 tgg2* double mutants produce very low amounts of aliphatic glucosinolate hydrolysis products, and tests on their crude extracts predictably showed that they receive decreased oviposition compared to Col-0 (Fig. 1a). In contrast, because the degradation of aliphatic glucosinolates is induced by physical foliage disruption, tests on intact plant

Table 1 Number of *Plutella xylostella* eggs laid in 24 h oviposition choice tests using commercially available indole glucosinolate breakdown products compared to solvent-only controls. Mean ± standard error, all comparisons for significance are Mann-Whitney *U*-tests

Breakdown Product	# of Eggs on Compound	# of Eggs on Solvent	*N*	*P*
Indole-3-Carbinol	0.150±0.11	1.45±0.52	20	*0.011
Indole-3-Acetonitrile	2.60±0.88	1.35±0.55	20	0.168
Indole-3-Carboxaldehyde	1.25±0.51	1.00±0.58	20	0.526
Indole-3-Carboxylic Acid	0.00±0.00	0.125±0.09	16	0.562
Diindolylmethane	0.579±0.37	0.316±0.15	19	0.783
Ascorbigen	0.500±0.35	0.050±0.05	20	0.534

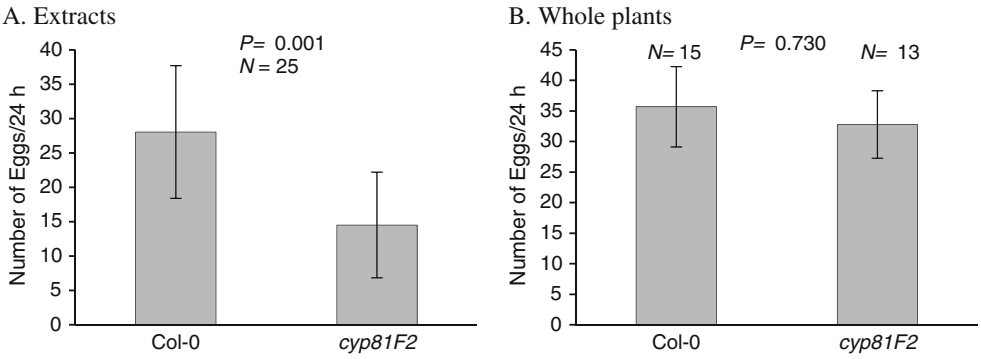

Fig. 5 *Plutella xylostella* 24 h oviposition choice tests comparing the number of eggs laid on wild-type Col-0 and mutants lacking 4M-I3M (*cyp81F2*). **a** Oviposition tests on crude plant extracts. **b** Oviposition tests on whole plants. Mean ± standard error. (a) significance determined by Wilcoxon signed rank tests. (b) significance determined by Mann-Whitney *U*-tests

tissue with little damage, i.e., whole rosettes and detached leaves, exhibited no such difference in oviposition between the mutants and wildtype (Fig. 1b, c) unless they were purposefully damaged (Fig. 1d). Alternatively, although less likely because of the above described results with the leaf extracts, the residual concentration of aliphatic glucosinolates (~6%), could be sufficient for *P. xylostella* attraction in whole plant experiments. The extremely low amount of isothiocyanates released in the headspace above undamaged crucifers (Finch 1978; Tollsten and Bergström 1988) does not appear to affect oviposition choices by *P. xylostella*, at least in the experiments described here.

Among the Lepidoptera, host plant selection by specialists through the detection of host-specific chemicals is well documented. In the monarch butterfly *Danaus plexippus*, a milkweed specialist, several tarsal and antennal contact chemorceptors detect non-volatile flavonoids found on the leaf surface of host plants, thus indentifying suitable oviposition targets (Haribal and Renwick 1996; Baur et al. 1998). Another lepidopteran crucifer specialist, *Pieris rapae,* also demonstrates a preference for ovipositing on *A. thaliana* containing intact indole glucosinolates and an aversion towards the indole glucosinolate breakdown product indole-3-acetonitrile (De Vos et al. 2008). Future experiments will determine the role of individual indole glucosinolates, including 4M-I3M, in *P. rapae* oviposition, thus allowing an interesting comparison with *P. xylostella*. For specialist insects, the identification of a suitable host plant confers a significant selective advantage by allowing them to take advantage of resources ill-suited for generalists. When reared on the mutant *A. thaliana* that lack components of the crucifer's defensive glucosinolate-myrosinase system, non-crucifer-specialist lepidopteran larvae experience a large increase in weight gain. This suggests that the glucosinolate content of wildtype plants has a significant negative effect on the fitness of these generalists (Barth and Jander 2006; Beekwilder et al. 2008; Schlaeppi et al. 2008).

Taking into the account the additive effect that intact indole glucosinolates and aliphatic glucosinolate breakdown products have in attracting oviposition by *P. xylostella,* as demonstrated with quadruple mutants that lack both indole and aliphatic glucosinolates (Fig. 2), these two types of compounds both appear to be important for *P. xylostella* attraction. Our experiments suggest that in the presence of indole glucosinolates, the effect of intact aliphatic glucosinolates is negligible (Fig 1b, c), but in the absence of indole glucosinolates, intact aliphatic or minute quantities of their breakdown products could account for attraction of *P. xylostella* females (Fig. 2b, d). Together, our data also suggest that intact indole glucosinolates are more reliable host recognition cues for ovipositing females on intact plants (Fig. 1c and d).

Perhaps, our experimental design, which used enclosed cages, directs *P. xylostella* host choice toward the use of tactile instead of olfactory cues. *Plutella xylostella* may recognize volatile isothiocyanates when in flight through olfaction by the antenna (Renwick et al. 2006), but nonvolatile intact indole glucosinolates are most likely detected at the leaf surface, possibly through leaf contact with tarsal chemoreceptors in a manner similar to that described for other Lepidoptera (Baur et al. 1998). We hypothesize that isothiocyanates and indole glucosinolates may function as long-range and short-range oviposition cues, respectively, for *P. xylostella*. The volatility of isothiocyanates allows for host recognition over a greater distance than molecules present at the leaf surface, but also decreases the reliability of detecting the molecule depending on plant and environmental conditions. Furthermore, because concentrations of isothiocyanates that affect *P. xylostella* oviposition are found only in extracts of *A. thaliana* plants (Fig. 1a), females that rely solely on this cue would risk decreased fitness of their offspring due to heightened plant defenses, increased presence of predators, and competition for nutrients by other insects on already damaged plants. The use of chemical host signals that results in the avoidance of the harmful effects that prior larval feeding would have on food availability, nutritional quality, and host defense responses has already been proposed for *P. rapae* (De Vos et al. 2008; Mumm et al. 2008) and *Pieris brassicae* (Rothschild and Schoonhoven 1977), and this concept may apply to *P. xylostella* as well. Interestingly, *P. xylostella* females preferred ovipositing on larvae-infested cabbage. Moreover, larval performance was

not impacted negatively by feeding conspecifics (Choh et al. 2008). These results might suggest that damage-induced aliphatic glucosinolate breakdown products function as host recognition cues without being associated with inherent negative effects on larval development. Future research aimed at understanding how the volatile and nonvolatile oviposition cues are recognized by specialist insects, such as *P. xylostella*, will lead to valuable insights to counteract these agriculturally important insect pests.

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mating by post-diapause winterforms begins in mid-February as ambient temperatures begin to increase (Krysan and Higbee 1990; Horton et al. 1998, 2007).

The role of chemical signals in psyllid mate location has only recently begun to be investigated. Behavioral evidence for a female-produced volatile sex attractant was reported for the pear psylla *C. bidens* by Soroker et al. (2004). More recently, similar results indicating male attraction to female-produced volatiles were obtained for both post-diapause winterform and summerform pear psylla, *C. pyricola* (Horton and Landolt 2007; Horton et al. 2007, 2008; Guédot et al. 2009), and for the citrus psyllid, *D. citri* (Wenninger et al. 2008).

Previous work with *C. pyricola* post-diapause winterforms showed that cuticular extracts of females were attractive to males (Guédot et al. 2009). These extracts were at least as attractive as a comparable number of live females, suggesting that cuticular extraction might be a suitable procedure for collecting the components of the attractant for analysis and identification (Guédot et al. 2009). Additionally, males were repelled by live males and extracts of males, further evidence that extracts contained sex-specific compounds or blends of compounds (Guédot et al. 2009). Thus, our first objective was to compare the chemical profiles of extracts from post-diapause winterform males and females, to look for differences between the sexes that might indicate putative pheromone components.

Olfactometer bioassays showed that field-collected winterform females did not become attractive to males until late February when ovarian maturation and mating begins in the field (Horton et al. 2007). Thus, our second objective was to compare the chemical profiles of extracts from post-diapause vs. diapausing winterforms. We were particularly interested in identifying chemicals that were exclusively or more abundantly present in post-diapause females than diapausing females, so as to pinpoint the chemicals that might be responsible for female attractiveness.

Several studies have suggested that, although psyllid males are attracted to female-produced pheromone, females are not attracted by volatiles from either sex. For example, females of the pear psylla *C. bidens* and the citrus psyllid *D. citri* did not exhibit attraction to either male or female conspecifics in bioassays (Soroker et al. 2004; Wenninger et al. 2008). With *C. pyricola*, field experiments that used live psylla indicated that females did not show a preference for either male- or female-baited traps compared to unbaited traps (Brown et al. 2009).

Our third objective was to determine if response by *C. pyricola* to a putative pheromone was sex-specific. To achieve this objective, we compared the behavioral responses of post-diapause males and females to an isolated chemical found to be more abundant in extracts of post-diapause females than post-diapause males. Because cuticular extracts of females were as attractive to males as live females (Guédot et al. 2009), we also compared attraction of males to the identified chemical vs. extracts of females, to determine whether this single chemical might be the major or even sole compound comprising the female-produced attractant.

For practical purposes, the development of a synthetic pheromone lure for pear psylla would provide a useful tool for monitoring and management strategies. Field experiments using live *C. pyricola* as attractants had shown that males exhibited a clear preference for female-baited traps vs. unbaited traps or traps baited with males (Brown et al. 2009). Thus, our final objective of this study was to field test the effectiveness of the chemical identified as a potential sex attractant for male pear psylla.

Methods and Materials

Source of Insects Winterform pear psylla were collected from a commercial pear orchard located near Yakima, Yakima Co., Washington, USA in December 2008 (diapausing winterform) and in February-March 2009 (post-diapause winterform) using a beat tray and aspirator. In December 2008, adults were separated by sex in the field, and placed in groups of ca. 350 insects on pear shoots (excised from branches in the field and placed in water vials) in 10-liter ventilated plastic containers. The containers and insects were stored at ~16°C under a L8:D16 photoperiod for 24 h before the insects were used for extractions. In February and March 2009, adults were separated by sex in the field and placed in groups of ca. 200 insects on pear shoots in 1-liter glass jars. The jars and insects were kept at ~24°C under a L16:D8 photoperiod for 72 h before the insects were extracted. Insects for olfactometer bioassays were held in glass jars under the same conditions for 3–7 d. On each collection date, a subsample of 10 post-diapause females collected in February-March 2009 was dissected to determine ovarian maturity (an indicator of diapause status), and the number of spermatophores (an indicator of the number of times a female had mated) (Krysan and Higbee 1990; Horton et al. 2007). Behavioral assays and extractions were not conducted until dissected females in the subsample had reached an average ovarian score of 5 or higher (Krysan and Higbee 1990), at which stage females are attractive to males in olfactometer assays (Horton et al. 2007). Although field collected psylla were mated, the mating status has no effect on female attractiveness in pear psylla (Horton et al. 2008).

Cuticular Extracts Extractions were performed between 12:00 and 15:00 h (P.S.T.). For each extraction, 50 psylla of one sex were transferred into an 11-ml glass vial containing 300 µl pentane for 5 min, during which the glass vial was agitated by hand. The solvent was then

transferred to a clean glass vial (extract). Simultaneously with each extraction, a control treatment was prepared using the same procedure with 300 µl pentane without psylla. Treatment and control extracts were stored for up to 5 d at 0°C until about 1 h before experiments were conducted. These samples were used in both chemical and behavioral assays. Samples used for chemical analyses were spiked with 10 µl of a 500-µg/ml solution of octadecane as an internal standard (Matheson Coleman & Bell, Cincinnati, OH, USA). In bioassays, each olfactometer consisted of paired 1-liter glass jars containing either the extract or a solvent control, applied with glass syringes (Hamilton Company®, Reno, NV, USA) to filter paper disks (55 mm diam; Whatman #1 Cat. No. 1001 055; Whatman®, Maidstone, UK), and allowed to evaporate in a fume hood for 1 min. Each filter paper disk was folded to prevent it from laying flat on the bottom of the jar. The disks were placed in the jars, and the jars were immediately attached to an olfactometer (described below).

Analysis of Extracts One-µl aliquots of extracts and associated solvent controls were analyzed by coupled gas chromatography-mass spectrometry (GC-MS) using an Agilent 6890 GC with a 5973 mass selective detector with electron impact ionization (70 eV). The GC was equipped with a DB-1MS fused silica capillary column, 0.25 mm ID×60 m, 0.25 µm film thickness (J&W Scientific, Folsom, CA, USA), programmed at 60°C for 1 min, increasing to 240°C at 30°C per min, then at 10°C per min to 340°C, and held for 13 min. Hydrocarbons were quantified using the integrated peak area data from the GC-MS response to increasing quantities (5–50 ng) of the authentic standards heptacosane, nonacosane, and hentriacontane (Sigma Aldrich®, St. Louis, MO, USA).

Quantitative GC-MS analyses were conducted to identify compounds present specifically or in greater abundance in females than males, comparing 1) diapausing winterform females with diapausing winterform males and 2) post-diapause winterform females with post-diapause winterform males. For each set, five extracts of females, five extracts of males, plus solvent controls, were analyzed. Because differences in chemical profiles between males and females of diapausing and post-diapause psylla appeared to be quantitative rather than qualitative, we calculated the female to male ratio for each identified chemical. Measurements of wing length and scutellum width of male and female pear psylla have shown that females are slightly larger than males, with a female to male ratio of less than 1.2 for both of these measurements (Wong and Madsen 1967). Thus, compounds that were at least twice as abundant in females compared to males were selected to be tested in the behavioral bioassays for attractiveness.

Compounds in extracts were identified by analysis on a Hewlett-Packard (HP) 6890 GC interfaced to a HP 5973 mass selective detector (electron impact ionization, 70 eV). The GC was fitted with a DB5-MS column (30 m× 0.25 mm i.d., 0.25 micron film thickness; J&W Scientific, Folsom, CA, USA), programmed from 100°C/1 min, 10°C/min to 280°C, and held for 20 min. One-µl aliquots were injected in splitless mode, with injector and transfer line temperatures at 280°C. Helium carrier gas was used in constant pressure mode.

Cuticular hydrocarbons were identified by a combination of retention time comparisons vs. straight-chain alkane standards (Carlson et al. 1998) and interpretation of their mass spectra. Where visible, the molecular ion indicated the total number of carbons in the molecule, methyl-branched hydrocarbons gave enhanced diagnostic ions at branch points that allowed the positions of the methyl branches to be determined, and the presence of methyl branches resulted in diagnostic shifts in retention times vs. straight-chain standards (Nelson 1993; Nelson and Blomquist 1995; Carlson et al. 1998). Long-chain aldehydes were identified tentatively by interpretation of their mass spectra and retention index comparisons with straight-chain alkanes, and identifications were confirmed where possible by comparisons of retention times and mass spectra with those of authentic standards.

Preparation of 13-Methylheptacosane Butyllithium (2.7 M in hexane, 1.1 ml, 3 mmol) was added to a slurry of tetradecyltriphenylphosphonium bromide (1.53 g, 2.8 mmol, Lancaster Research Chemicals, Windham, NH, USA) in 20 ml anhydrous ether under argon at room temperature. After stirring for 30 min, a solution of 2-tetradecanone (0.30 g, 1.4 mmol) in 5 ml ether was added, the resulting slurry was stirred 30 min, then poured into 1 M aqueous HCl. The mixture was extracted with hexane, and the hexane extract was washed with saturated aqueous $NaHCO_3$ and brine, then dried over anhydrous Na_2SO_4 and concentrated. The residue was taken up in ~10 ml hexane, and left overnight to precipitate the bulk of the triphenylphosphine oxide. The resulting solution was purified by vacuum flash chromatography on silica gel, eluting with hexane. The mixture of purified alkenes in hexane was then hydrogenated with 100 mg of 5% palladium on charcoal catalyst in a septum-sealed flask fitted with a balloon full of hydrogen for 3 h. After filtration, the crude product (0.43 g, ~95% pure by GC) was recrystallized from 12 ml acetone at −20°C, yielding 0.21 g 13-methylheptacosane as a low-melting white solid, mp 26°C. The ^{1}H NMR spectrum matched that previously reported (Marukawa et al. 2001). The mass spectrum showed *m/z* (abundance) 394 (trace, M^+), 379 (1), 365 (1), 225 (8), 224 (12), 197 (9), 196 (19), 168 (4), 155 (5),

141 (7), 127 (10), 113 (15), 99 (22), 85 (62), 71 (80), 57 (100), 43 (53).

Behavioral Bioassays A Y-tube olfactometer was used to assess the response of male and female post-diapause winterform psylla to olfactory signals. The olfactometer, fully described in Horton and Landolt (2007), consisted of a 27-cm long, 2.5 cm diam glass tube forming the stem of the Y, joined to two arms at 135° to one another, each 7 cm in length. The Y-tube was positioned horizontally with a ~15° incline. Commercial compressed air (Oxarc Inc., Spokane, WA, USA) was passed through a charcoal filter, an air humidifier, and 1-liter glass jars containing odor sources. A 25 cm × 2 mm diam polytetrafluoroethylene hose (PTFE) (Cole-Parmer Instrument Company, Vernon Hills, IL, USA) connected each jar to an arm of the Y-tube. Airflow through each arm of the olfactometer was maintained at 50 ml per min during the assays. Before each bioassay, air was passed through the whole system at 50 ml/min in each arm, including the jars containing the odor sources, for 15 min. For all assays, a replicate consisted of 10 psylla of the same sex assayed individually. For each replicate, after five psylla were assayed, the arms of the olfactometer were rotated 180° horizontally, and the other 5 males were assayed. Approximately 30 min preceding an assay, psylla to be assayed were placed in a 50 ml vial. During the assays, a single psylla was allowed to enter the olfactometer from the vial and was then given 10 min to enter an arm of the Y-tube. Psylla that did not enter an arm within 10 min were discarded. Choice was defined by a psylla contacting the upwind end of an arm, at the insertion point of the PTFE hose. After each replicate, the olfactometer was dismantled, soaked in hot soapy water, rinsed with water, acetone and hexane, and then baked in an oven at 150°C for at least 2 h. Two sets of assays were carried out, with comparisons within each set being randomized.

Bioassays were conducted using the Y-tube olfactometer to address response of post-diapause winterform male and female psylla to 13-methylheptacosane [hereafter, 13-MeC27], which was found in the chemical analyses to be substantially more abundant in extracts from post-diapause females than post-diapause males. In a first experiment, two comparisons were made in which either males or females were given a choice between 500 ng of 13-MeC27 and solvent controls. Each comparison consisted of 10 replicates. In a second experiment, three comparisons were conducted of post-diapause males to 13-MeC27 vs. cuticular extracts of post-diapause females: (a) 13-MeC27 vs. control; (b) cuticular extract of females vs. control; and (c) 13-MeC27 vs. cuticular extract of females. Each comparison consisted of 15 replicates. In the first and third comparisons, 250 ng of 13-MeC27 (equivalent to ~25 females) were applied to a filter paper. Each cuticular extract consisted of 25 females extracted in pentane for 5 min.

Field Trial A field assay was conducted 2–7 April 2009 in a pear psylla-infested pear orchard at the United States Department of Agriculture's Experimental Farm near Moxee, Yakima Co., WA, USA (46°30′ 18.60″ N 120°10′ 06.64″ W). The orchard is a mixture of Bartlett and Anjou pear trees approximately 3 m in height, with ~3 m spacing between tree trunks. Sticky traps were used to assess the attraction of male and female winterform pear psylla to 13-MeC27. Each trap was constructed of a 30×30 cm section of light brown Lumite® fabric (No. 5006304, Chicopee Mills Inc., New York, NY, USA) with 1 mm^2 sized mesh. Two opposite edges of a fabric section were each stapled to a 30 × 1.5 × 0.5 cm piece of hemlock. A ~30 cm section of twisty tie was stapled to the center of each hemlock piece, and traps were hung between two branches using the twisty ties. The fabric section was coated with a thin layer of Tanglefoot Tangle-Trap Sticky Coating (Grand Rapids, MI, USA). Gray halobutyl rubber septa (West Pharmaceutical Services, Lyonville, PA, USA) were used as dispensers. Septa were pre-extracted with methylene chloride, and then aired for 24 h at 24°C in a fume hood before use. Septa were loaded with 10, 100, or 1,000 μg of 13-MeC27 in 100 μl aliquots of pentane, followed by an additional 200 μl of pentane to adsorb the test compound into the rubber matrix. Control lures were treated with 100 μl of pentane, followed by 200 μl of pentane. Lures were aired at room temperature in a fume hood overnight, then stored in a freezer at 0°C overnight until use in field traps. Lures were attached to the center of traps using safety pins. Treatments were deployed in a randomized complete block design with eleven blocks. Each block consisted of four treatments: control, and 10, 100, and 1,000 μg doses of 13-MeC27 per septum. Traps were placed one per tree at ~1.5 m above ground; two trees separated adjacent traps. Traps were collected from the field on 7 April, and returned to the laboratory where captured male and female psylla on each trap were counted.

Statistical Analyses Statistical analyses were performed using SAS Version 9.1 for Windows (SAS Institute 2002). Behavioral bioassay data were analyzed with paired sample *t*-tests in PROC TTEST as described in Horton et al. (2007, 2008). The *t*-test assumes a normal distribution of the arithmetic differences between paired observations (Zar 1999). The normality assumption was tested using the Shapiro-Wilk statistic in PROC UNIVARIATE. When the normality assumption was not met, the paired differences were analyzed using a signed-ranks test in PROC UNIVARIATE (Zar 1999; Horton et al. 2007, 2008). Because the signed-ranks test yielded significant differences in all comparisons, possibly leading to the commission of a type I error, we opted for a more conservative approach and used results obtained with paired sample *t*-tests.

For the field assays, count data were square-root transformed to normalize the distributions for analysis. The data were analyzed as a randomized block, two-factor (sex × treatment) repeated measures analysis of variance using PROC MIXED, with sex being the repeated factor because both sexes were counted on each trap. The means of the three doses (10 μg, 100 μg, and 1,000 μg) were compared to the control mean to assess whether the compound led to an increase in trap catches vs. the unbaited controls. In the event of a significant treatment by sex interaction, the SLICE statement in PROC MIXED was used to examine treatment effects for each sex separately. Following a significant *F* value for the ANOVA, differences between means were separated by Tukey's test.

Results

Analysis of Extracts Analyses of cuticular extracts from male and female winterform *C. pyricola* revealed the presence of a number of long chain hydrocarbons and related compounds (Fig. 1; Tables 1 and 2). The identified cuticular hydrocarbons of winterform pear psylla comprised 36.4±1.8% (mean ± S.E.M.) C_{23}-C_{35} straight-chain alkanes, 28.8±0.5% C_{25}-C_{33} monomethyl-branched alkanes, 7.1± 0.6% C_{31} dimethyl-branched alkanes, and 15.0±0.4% C_{22}-C_{30} aldehydes. The most abundant compounds found in all extractions were heptacosane, 2-methylheptacosane, nonacosane, 2-methylnonacosane, hentriacontane, and a mixture of 11,15- and 13,17-dimethylhentriacontanes.

Comparisons of the chemical profiles of males and females of either diapausing or post-diapause psylla revealed quantitative rather than qualitative differences, with every chemical identified being present, although in different amounts, in every extract. Total amounts of identified hydrocarbons were significantly lower in diapausing vs. post-diapause winterforms (t=4.1; df=18; P<0.001), varying from 528.8±21.7 (mean ± S.E.M.) ng and 581.7±36.3 ng for diapausing males and females, respectively, to 690.6± 47.0 ng and 712.7±36.0 ng for post-diapause males and females, respectively. Diapausing winterforms had no chemicals that were at least twice as abundant in females as in males. By contrast, in post-diapause winterforms, 13-MeC27 was 3.2 times more abundant in females than males. The 13-MeC27 was also the most abundant compound in diapausing females compared to diapausing males, although it was only 1.5 times more abundant in females than males.

Three chemicals, 13-MeC27, tetracosanal, and hexacosanal, were at least two times more abundant in post-diapause females than diapausing females. Two of these chemicals (tetracosanal and hexacosanal) were also more abundant in post-diapause males compared to diapausing males.

Behavioral Bioassays In the first experiment on the response of post-diapause winterform males and females to 13-MeC27, all 100 males and 100 females assayed made a choice within the 10 min cutoff time. Males most often chose the filter paper that had been treated with the 13-MeC27 when paired with a solvent control filter paper; 62.0% of males choosing the 13-MeC27 (t=4.81; df=9; P=0.001; Fig. 2a). Females showed no preference when presented with the 13-MeC27 vs. the solvent control (t=1.18; df=9; P=0.27; Fig. 2a).

In the second experiment on the response of winterform males to 13-MeC27 vs. cuticular extract of winterform females, all 450 males assayed made a choice within 10 min. Males most often chose the filter paper treated with the 13-MeC27 when it was paired with a solvent control filter paper; 67.3% of males chose the 13-MeC27 (t=11.31; df=14; P<0.0001; Fig. 2b). Similar results were obtained when assaying males to filter papers treated with extracts of females vs. solvent controls; 63.3% of males chose the extract of females (t=5.74; df = 14; P<0.001; Fig. 2b). Males did not show a preference when exposed to the 13-MeC27 vs. the extracts of females, with 50.7% of males selecting the 13-MeC27 (t=0.27; df=14; P=0.79; Fig. 2b).

Field Trial A total of 6,974 male and 3,958 female psylla were caught over the 6 d of the study. A significant interaction between sex and treatment was observed (F=8.76; df=3, 70; P<0.001) (Fig. 3). The mean catch of females on traps baited with 13-MeC27 was not significantly different from catch on the control traps, regardless of the dose of 13-MeC27 (F=0.25; df=3, 70; P=0.86). In contrast, the mean trap capture of males was higher on traps baited with 13-MeC27 than control traps (F=20.31; df=3, 70; P<0.001) (Fig. 3). The control traps did not capture more males than females (F=1.49; df=1, 70; P=0.23). More males than females were caught on traps baited with the 10 μg dose (F= 21.58; df=1, 70; P<0.001), with the 100 μg dose (F=28.95; df=1, 70; P<0.001), and with the 1,000 μg dose (F=6.79; df=1, 70; P=0.01). Furthermore, more males were caught on traps baited with the 10 μg dose (t=-6.28; df=70; adjusted P<0.001), with the 100 μg dose (t=−7.15; df=70; adjusted P<0.001), and with the 1,000 μg dose (t=−4.69; df=70; adjusted P<0.001) than on the control traps.

Discussion

The role of chemical signals in mate location is well established for many species in numerous insect orders. Recently, studies have shown that males are attracted to female-produced volatiles in three species of Psyllidae: *C. bidens* (Soroker et al., 2004), *C. pyricola* (Horton and

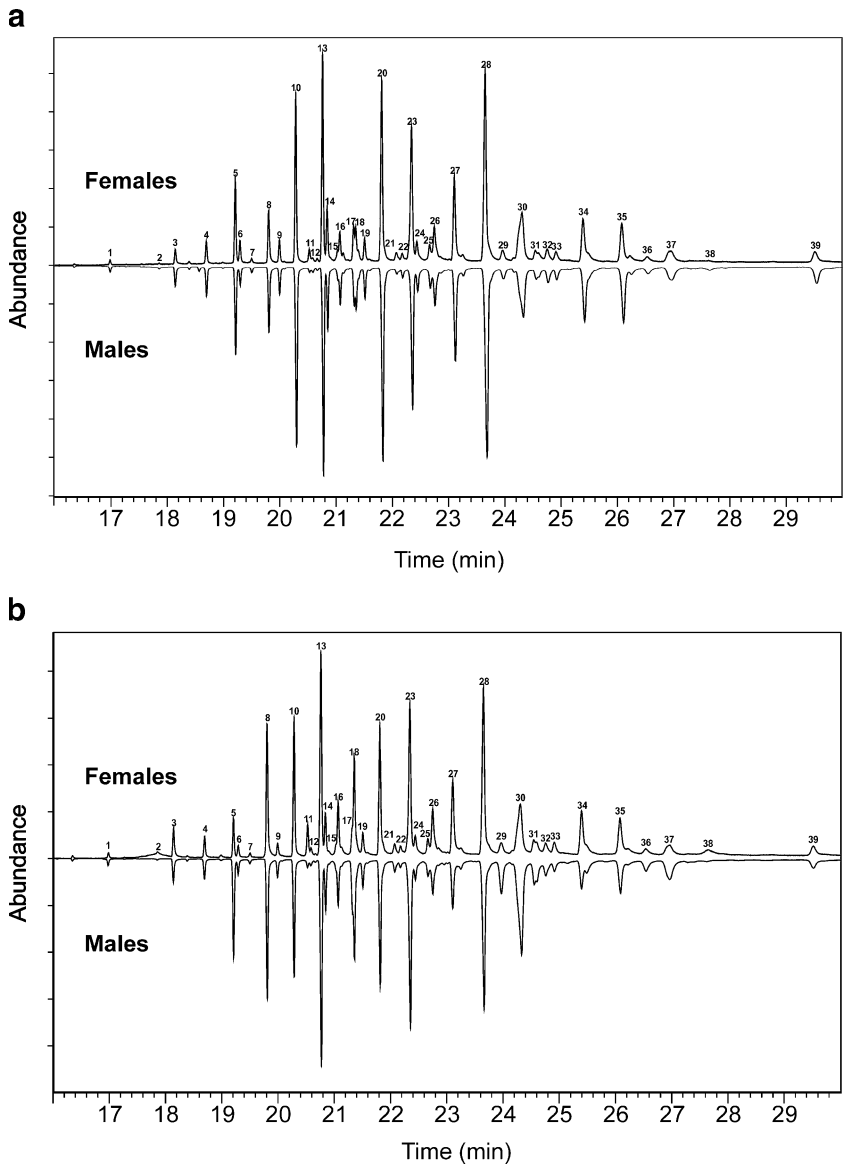

Fig. 1 Gas chromatography-mass spectrometry (GC-MSD) profiles of representative solvent extract of (**a**) diapausing winterform females (upper trace) and males (lower trace) and (**b**) post-diapause winterform females (upper trace) and males (lower trace), run on the DB5-MS column. Identifications of the numbered peaks are reported in Table 1

Landolt, 2007; Horton et al., 2007, 2008; Guédot et al. 2009), and *D. citri* (Wenninger et al., 2008). Despite the evidence and interest in the role of olfactory signals in mate location in the Psyllidae, the chemical(s) involved in the sex attractants had not been identified for any of these species.

The primary role of insect cuticular hydrocarbons is to provide a hydrophobic barrier that minimizes transpiration and that prevents desiccation (Nelson 1978; Lockey 1988; Howard 1993). However, components of the cuticular lipids often have important secondary roles as intraspecific recognition signals that communicate information such as sex, species, and physiological state (for review see Singer, 1998; Howard and Blomquist 1982, 2005). In the present study, the chemical analysis of cuticular extracts of male and female winterform *C. pyricola* revealed the presence of long chain (C22 to C35) *n*-alkanes, methyl- and dimethyl-branched alkanes, and aldehydes. To our knowledge, this is the first study that describes the cuticular lipid composition of any species in the Psyllidae.

The chemical profiles of extracts from diapausing and post-diapause winterforms were similar, with quantitative rather than qualitative differences. Post-diapause winterforms had greater amounts of cuticular hydrocarbons than diapausing winterforms. Similarly, hydrocarbon profiles of the adult face flies *Musca autumnalis* De Geer indicated a small increase in the total amount of hydrocarbons on the cuticle of reproductive females compared to diapausing females (Jurenka et al. 1998). In contrast, diapausing mosquitoes, *Culex pipiens* Linnaeus, produced more cuticular hydrocarbons than non-diapausing mosquitoes, purportedly as a mechanism to reduce water loss (Benoit and Denlinger 2007). Overall, the diapause syndrome is a

Table 1 Identification of hydrocarbons and related compounds in cuticular extracts of *Cacopsylla pyricola*. Identifications of compounds in boldface type have been confirmed by matches of retention indices and mass spectra with those of authentic standards

Peak #[a]	Retention index[b]	Identification	Diagnostic ions[c]
1	2300	**tricosane**	324 [M^+]
2	2400	**tetracosane**	338 [M^+]
3	2435	**docosanal**	278, 306 (324, M^+)
4	2500	**pentacosane**	352 [M^+]
5	2563	2-methylpentacosane	323, 351 (366, M^+)
6	2581	3-methylpentacosane	337 (366, M^+)
7	2600	**hexacosane**	366 [M^+]
8	2638	**tetracosanal**	306, 334, 352 [M^+]
9	2663	2-methylhexacosane	337, 365 (380, M^+)
10	2700	**heptacosane**	380 [M^+]
11	2736	**13-methylheptacosane**	196/224 (394, M^+)
12	2740	**pentacosanal**	348 (366, M^+)
13	2764	2-methylheptacosane	351, 379, 394 [M^+]
14	2775	3-methylheptacosane	365, 379 (394, M^+)
15	2800	**octacosane**	394 [M^+]
16	2804	unidentified	
17	2835	unidentified	
18	2842	**hexacosanal**	334, 362, 380 [M^+]
19	2861	2-methyloctacosane	365, 393 (408, M^+)
20	2900	**nonacosane**	408 [M^+]
21	2931	11-, 13- and 15-methyl-nonacosane	168/280; 196/252; 224, (422, M^+)
22	2943	**heptacosanal**	376 (394, M^+)
23	2963	2-methylnonacosane	379, 407 (422, M^+)
24	2973	3-methylnonacosane	393 (422, M^+)
25	3000	**triacontane**	422 [M^+]
26	3012	unidentified	
27	3044	**octacosanal**	362, 390, 408 [M^+]
28	3100	hentriacontane	436 [M^+]
29	3128	11-, 13- and 15-methyl-hentriacontane	168/308; 196/280; 224/252, (450, M^+)
30	3159	11,15- and 13,17-dimethyl-hentriacontane	168/239, 252/323 (464, M^+); 196/267, 224/295 (464, M^+)
31	3181	unidentified	
32	3186	unidentified	
33	3200	**dotriacontane**	450 [M^+]
34	3248	triacontanal	390, 418, 436 [M^+]
35	3300	tritriacontane	464 [M^+]
36	3329	11-methyltritriacontane	168/336 (478, M^+)
37	3356	unidentified	
38	3400	**tetratriacontane**	478 [M^+]
39	3500	pentatriacontane	(506, M^+)

[a] Numbers correspond to peaks in chromatograms in Fig. 1a and b.

[b] Retention indices calculated versus straight chain alkanes on DB5-MS column.

[c] [M^+] indicates a visible molecular ion of the mass shown. Values in round brackets indicate molecular ions which were not visible, but which could be inferred from the diagnostic fragments and the retention time.

complex and often poorly understood process in which changes in cuticular lipids form but one small part of the overall changes in the physiological state of an insect.

The sexual dimorphism observed in the cuticular lipid profiles in both diapausing and post-diapause winterforms was quantitative rather than qualitative, with the same hydrocarbons being present in both sexes for both diapause states. Comparisons of the relative amounts of cuticular hydrocarbons in the post-diapause winterform revealed that only one chemical, 13-methylheptacosane (13-MeC27), was at least two times more abundant in females than males. Furthermore, comparisons of winterforms by sex revealed that post-diapause females produced relatively larger amounts of two other compounds, tetracosanal and

Table 2 Mean (± S.E.M.) amounts (ng) of cuticular hydrocarbons and related compounds per psylla for females and males of the diapause and post-diapause winterform of *Cacopsylla pyricola*, and female to male ratio (F:M) for each hydrocarbon. Peak in boldface type is 13-methylheptacosane. *N*=5 replicates

Peak #[a]	diapausing winterform			post-diapause winterform		
	female	male	F:M[b]	female	male	F:M
1	4.1±0.2	3.8±0.1	1.1	4.3±0.3	4.3±0.2	1.0
2	t[c]	t	n/a[d]	t	t	n/a
3	4.1±0.2	3.7±0.1	1.1	4.6±0.3	4.8±0.4	1.0
4	6.5±0.5	6.0±0.4	1.1	6.4±0.4	6.2±0.4	1.0
5	13.7±0.9	11.9±0.7	1.2	9.8±0.9	17.2±1.2	0.6
6	6.3±0.3	4.7±0.2	1.3	4.8±0.2	4.6±0.2	1.1
7	3.5±0.1	3.4±0.0	1.0	t	t	n/a
8	7.7±0.7	6.3±0.4	1.2	18.8±2.0	22.6 ± 2.4	0.8
9	6.3±0.5	6.2±0.2	1.0	5.6±0.3	5.7±0.3	1.0
10	31.6±3.1	28.9±2.7	1.1	30.9±2.0	25.9 ± 2.0	1.2
11	**4.9±0.3**	**3.3±0.1**	**1.5**	**11.5±0.9**	**3.6±0.2**	**3.2**
12	3.0±0.0	3.0±0.0	1.0	2.9±0.0	2.7±0.0	1.1
13	34.0±1.8	31.5±1.8	1.1	50.6±3.8	43.7±3.5	1.2
14	11.2±0.5	10.7±0.6	1.0	12.3±0.8	10.7±0.7	1.1
15	7.5±0.2	7.2±0.1	1.0	5.3±0.2	4.5±0.2	1.2
16	6.1±0.4	5.3±0.2	1.2	9.5±0.8	8.3±0.8	1.1
17	10.9±0.4	10.1±0.5	1.1	12.9±0.6	14.7±0.7	0.9
18	8.8±0.5	7.9±0.4	1.1	21.4±1.8	22.0 ± 1.9	1.0
19	8.9±0.3	9.5±0.3	0.9	11.1±0.4	11.1 ± 0.5	1.0
20	39.3±2.7	37.0±2.1	1.1	39.4±2.3	34.9 ± 2.3	1.1
21	6.9±0.3	6.2±0.1	1.1	9.7±0.2	8.1 ± 0.3	1.2
22	6.2±0.1	6.1±0.1	1.0	7.2±0.2	6.8±0.2	1.1
23	32.6±1.7	29.7±1.2	1.1	49.5±2.6	47.2 ± 4.0	1.0
24	9.0±0.2	9.0±0.2	1.0	10.9±0.4	10.0±0.6	1.1
25	9.0±0.3	8.7±0.2	1.0	11.1±0.40	10.3±0.5	1.1
26	10.3±0.5	8.4±0.2	1.2	13.9±0.8	11.2 ± 0.7	1.2
27	28.0±3.1	23.1±1.9	1.2	29.6±2.4	26.2 ± 1.8	1.1
28	56.9±3.3	53.4±3.0	1.1	69.0±4.1	60.0±4.5	1.1
29	14.4±0.9	13.8±0.6	1.0	19.7±1.1	25.0 ± 1.7	0.8
30	37.8±2.4	31.9±1.4	1.2	50.4±2.9	61.3±5.6	0.8
31	13.5±0.6	12.9±0.5	1.0	19.6±1.1	20.1 ± 2.0	1.0
32	10.7±0.3	10.3±0.2	1.0	15.4±0.4	15.8±0.6	1.0
33	17.2±1.2	17.5±1.0	1.0	24.2±1.2	22.5 ± 2.3	1.1
34	26.7±4.0	24.1±2.2	1.1	26.9±2.7	24.1 ± 1.8	1.1
35	28.4±3.2	24.9±1.8	1.1	30.7±1.9	27.3 ± 2.1	1.1
36	13.0±1.2	12.3±1.0	1.1	15.4±1.0	17.1 ± 1.4	0.9
37	19.8±2.2	15.5±1.1	1.3	20.7±1.5	24.0 ± 2.1	0.9
38	8.5±0.0	8.7±0.0	1.0	11.2±0.1	11.5±0.1	1.0
39	13.2 ± 0.7	11.8±0.3	1.1	15.4±0.5	14.5±0.4	1.1

[a] Numbers correspond to peaks in Fig. 1

[b] Female to male ratio

[c] t = trace amounts (<0.5 ng per injection)

[d] n/a = not applicable

hexacosanal, compared to diapausing females, while post-diapause males produced relatively larger amounts of the two aldehydes than diapausing males. The two aldehydes were found in similar abundance in post-diapause females and males and are, therefore, probably not involved in mate attraction. Furthermore, in the Y-tube assays, 13-MeC27 alone was as attractive to males as the extract of females, which contained 13-MeC27 and a number of other hydrocarbons, including tetracosanal and hexacosanal. These results suggest that the relative and possibly absolute amount of 13-MeC27 produced by post-diapause females plays an important role in female attractiveness. Although 13-MeC27 is not specific to females, similar results have been obtained with other insects. For example, the contact sex pheromone produced by females of the locust borer, *Megacyllene robiniae* (Förster), also

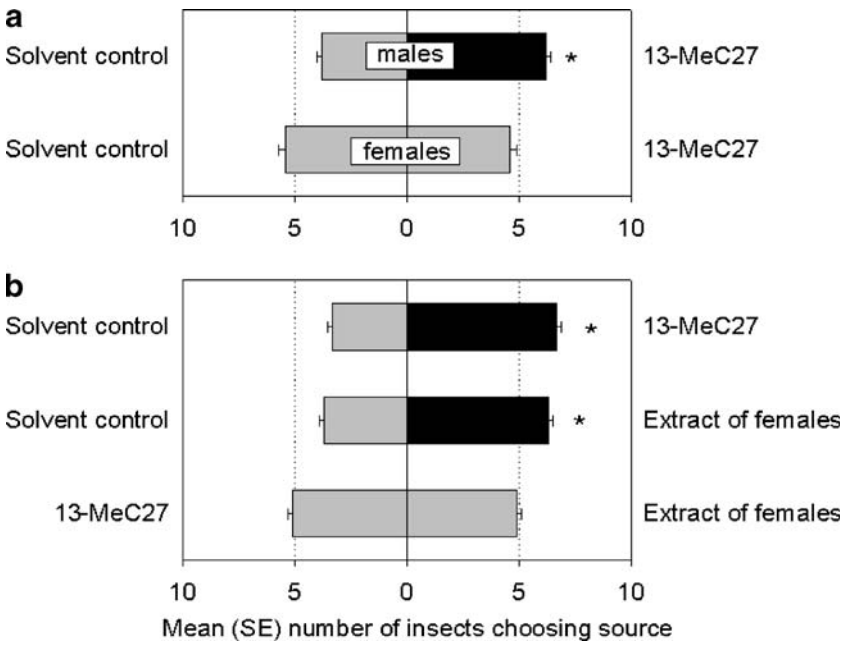

Fig. 2 Numbers of post-diapause winterform psylla (mean + S.E.M.) that chose paired odor sources. (**a**) Responses of males and females to 500 ng of 13-methylheptacosane (13-MeC27) vs. a solvent control (N=10). (**b**) Response of males to: 13-MeC27 (250 ng) vs. solvent control; extract of females (25 females) vs. solvent control; 13-MeC27 (250 ng) vs. extract of females (25 females) (N=15 per bar). * Choice is significant at P<0.05

was present, although in smaller quantities, on males (Ginzel et al. 2003).

The behavioral bioassays showed that responses to 13-MeC27 were sex-specific, with only males being attracted in both laboratory and field bioassays. In these assays, we tested the racemic blend of 13-MeC27, leaving male and female response to the individual stereoisomers unaddressed. The lack of responses by females to either female- or male-produced volatiles was also observed in the field with *C. pyricola* (Brown et al. 2009), and in the laboratory with *C. bidens* (Soroker et al. 2004) and *D. citri* (Wenninger et al. 2008). Males did not prefer the female extract over an equivalent amount of 13-MeC27, suggesting that 13-MeC27 may have been solely responsible for female attractiveness. These behavioral results are consistent with results described in the chemical analyses, and support the hypothesis that 13-MeC27 is a sex attractant pheromone for post-diapause males in *C. pyricola.* Further studies should address the attractiveness of the chiral forms of 13-MeC27.

13-Methylheptacosane is a relatively common constituent of insect lipids, and has been previously reported as a pheromone in insects. It is the major component of the contact recognition pheromone in males of the butterfly, *Colias eurytheme* (Boisduval) (Grula and Taylor 1979), and may be an aphrodisiac pheromone in that species (Grula et al. 1980). A mixture of three mono-methylheptacosanes, including 13-MeC27, was described as the major component of the post-pharyngeal gland secretions in the harvester ants *Pogonomyrmex salinus* Olsen and *Messor lobognathus* Andrews (Do Nascimento et al. 1993). As a cuticular hydrocarbon, 13-MeC27 occurs in the flesh fly, the pecan weevil, ants, grasshoppers, and crickets (reviewed in Nelson, 1978), and in termites (Haverty et al. 1996), wasps (Singer et

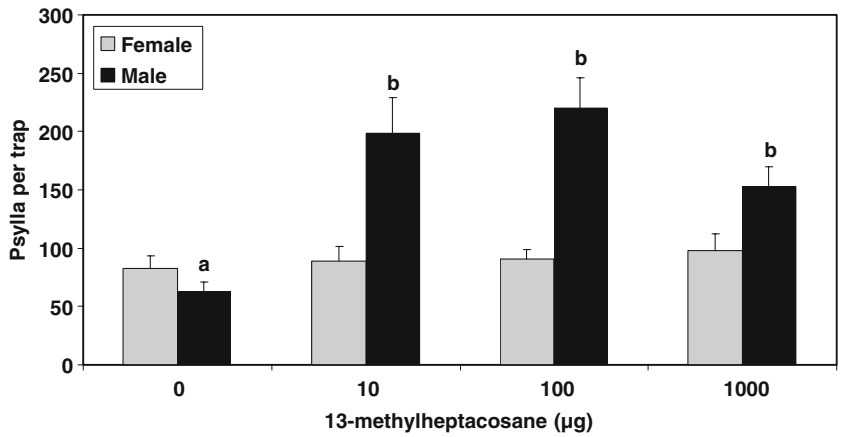

Fig. 3 Mean (+ S.E.M.) number of female and male winterform pear psylla captured on traps baited with 13-methylheptacosane dispensed from gray rubber septa at doses ranging from 0 to 1,000 µg (N=11 traps per dose). For male trap catches, treatments with different letters above them are significantly different (Tukey test, adjusted P≤0.05). Test conducted from 2 to 7 April 2009 near Moxee, WA

al. 1992; Liepert and Dettner 1996), and the moth *Scoliopteryx libatrix* (Linnaeus) (Subchev and Jurenka 2001).

Post-diapause winterform males of *C. pyricola*, but not females, were attracted to 13-MeC27 in the field. These results support field results with pear psylla indicating male attraction to females and no attraction between males, between females, and females to males (Brown et al. 2009), and support results of laboratory behavioral bioassays. This is also consistent with findings in two other psyllid species (Soroker et al. 2004; Wenninger et al. 2008). Our results indicate that males are attracted to a range of doses of 13-MeC27 covering two orders of magnitude, and that the doses tested were not critical for male attraction.

In summary, our results suggest that 13-MeC27 is a sex attractant pheromone for *C. pyricola* winterform males. Sex attractants are known to occur in non-psyllid Sternorrhyncha, including aphids, mealybugs, scale insects, and white flies (Yin and Maschwitz 1983; Lanier et al. 1989; Pickett et al. 1992; Hinkens et al. 2001; Rodriguez et al. 2005). This study provides the first identification of a sex pheromone in the Psylloidea. Further studies should address the optimization of this sex attractant for integrated pest management applications. Potential applications include a lure and trap useful to pear growers for monitoring pear psylla, lure and kill technologies, and mating disruption strategies for pear psylla management.

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Prior to this research, laboratory studies that used a two choice olfactometer found that male *A. erythrocephala* exhibit a series of characteristic behaviors in response to female derived volatiles, as well as to decanal. The latter can be obtained from aerations and whole body extracts of both sexes (Staples 1999). Male behaviors included antennation, upwind flight, and courtship behavior that involved abdominal flexing accompanied by substrate vibration (Staples 1999). These responses were similar to laboratory and field data from another Pamphilliid sawfly, the web-spinning larch sawfly, *Cephalcia lariciphila*, in response to female derived odors and to a likely pheromone component, ortho-aminoacetophenone (Borden et al. 1978; Baker et al. 1983).

In this paper, we report that female *A. erythrocephala* produce an attractant pheromone, (*Z*)-6,14-pentadecadienal, to which only males respond. We demonstrate that the synthetic compound attracts male sawflies in the field, and provide preliminary evidence indicating that (*Z*)-6,14-pentadecadienal can be produced via abiotic oxidation of a long-chain unsaturated hydrocarbon found exclusively in females.

Methods and Materials

Life History In North America, adult *Acantholyda erythrocephala* emerge from underground in spring (March to May depending on latitude) to mate and oviposit (MiddleKauff 1958; Lyons 1994; Asaro and Allen 1999). Flight season lasts for approximately 3 wk, depending on local climate and weather conditions. Females oviposit on the previous year's needles. Larvae hatch approximately 10 d later and feed gregariously from within protective webs for 18–21 d (Middlekauff 1958). In May to July (depending on latitude), last-instars drop to the forest floor and burrow 1–15 cm into the soil (Jahn 1967; MiddleKauff 1938; Asaro and Allen 1999). Larvae overwinter in earthen cells as bright green pronymphs. Pupation occurs in the following spring (Jahn 1967), followed by adult emergence. Adult *A. erythrocephala* are strongly phototropic and readily bask in direct sunlight on cool days. As such, population densities usually are greater along sunny forest edges or in well-lit patches on the forest floor.

Collection and Rearing of Live Insects Most *A. erythrocephala* used in this research were collected as pronymphs in Sand Ridge State Forest (Mason County, IL, USA) during fall 2001, 2002, and 2003, by digging with a shovel under defoliated trees to a depth of approximately 15 cm. The bright green pronymphs were identified readily and collected by hand. Pronymphs were transported to the laboratory and placed in 31×8×6 cm plastic trays, 100–200 per tray, on moist, silica sand, 3 cm to 4 cm deep. Trays containing insects were then placed in growth chambers under total darkness at 4°C and ~70% RH for 2 mo. Pupation was initiated by raising the temperature to 12°C for 10–12 d.

Pupae and pre-emergent adults also were collected from the Sand Ridge site in mid March through early April and used for chemical analyses, behavioral studies, and field tests. Some adults were kept in 26×39×23 cm plastic boxes fitted with aluminum screen covers and maintained in the dark at 4°C and ~70% RH. Under these conditions, groups of healthy male or female sawflies (200 individuals per box) could be kept alive for up to 30 d.

Volatile Collection Volatiles were collected from aerations of individual and multiple male or female sawflies at the National Center for Agricultural Utility Research, Peoria, IL, as per Cossé and Bartelt (2000) and Cossé et al. (2001). Sawflies were aerated in 150 ml glass tubes (3×30 cm), and the resulting volatiles were collected on Super Q traps (80–100 mesh, Alltech Deerfield, IL, USA) (1×0.5 cm diam) plugged with silanized glass wool. These were placed at the inlet and outlet of each chamber. Airflow was held at 50 ml min^{-1} via valve-controlled vacuum. Sawflies were given access to water in 2 ml glass vials plugged with cotton and placed inside each chamber. Volatiles were collected from 1 d to 4 d under florescent light at 27°C and a 16 L:8D photoperiod. Outlet traps from each aeration were rinsed with 500 µl of HPLC grade hexane (Fisher Scientific) and concentrated to 50 µl under a steady stream of nitrogen and stored at −70°C.

Whole Body Extracts Whole body extracts were obtained from adult *A. erythrocephala* by soaking groups of six male or six female sawflies in 2 ml hexane for 2 min. The extracts then were concentrated to 200 µl under a stream of nitrogen. A series of fractions (1.5 ml of each) of increasing polarity were eluted on a silica gel column (0.6 cm ID× 2 cm height, gravity flow), beginning with, hexane, followed by 5%, 10%, and 25% diethyl ether (redistilled) in hexane, and finally 100% ether. Fractions were concentrated to 30 µl under a stream of nitrogen, and analyzed as described below for specific chemical constituents.

Chemical Analysis Volatile collections and chromatographic fractions of whole body washes were analyzed by using coupled gas chromatography-mass spectrometry (GC-MS). Equipment consisted of a Hewlett-Packard model 6890 gas chromatograph interfaced with a Hewlett-Packard model 5973 mass selective detector. Injections were made through a split/splitless inlet, operated in splitless mode. Columns used included DB-5 (30×0.25 mm ID with 0.25 µm film, J&W Scientific, Folsom, CA, USA), DB-1 (15×0.25 mm ID with 0.10 µm film, J&W Scientific), and a 30 m EC-5

(0.25 mm ID, 0.25 μm film thickness, Alltech Associates, Deerfield, IL, USA). A typical temperature program was 50°C for 1 min, increasing at 10°C min^{-1} to 300°C, and with a final hold time of 20 min. GC-MS settings were modified as necessary for specific analyses; however, the mass spectrometer scanning range was typically 40 amu to 550 amu (EI, 70 eV), except in analysis of dimethyl disulfide (DMDS) derivatives where the maximum mass was increased to 650 amu. The 1995 version of the Wiley mass spectral library was available on the data system (Wiley 1995).

Dimethyl disulfide (DMDS) derivatives of the unsaturated cuticular hydrocarbons were prepared and analyzed to determine double bond locations. The hexane fractions of the male and female whole body washes were prepared and analyzed according to the method in Carlson et al. (1989). Synthetic standards for (*Z,Z*)-1,9,15-pentacosotriene, (*Z*)-9,15-pentacosodiene, (*Z*)-9-pentacososene, and 6-14-pentadecadienal, were analyzed likewise to further verify chemical structures. GC-MS of DMDS derivatives was carried out on a thin film (0.1 μm) DB-1 column. GC inlet and transfer lines were set to 230°C and mass scan upper limit was set to 650 amu.

Electrophysiology Aerations and polar fractions of whole body extracts were analyzed by gas chromatography coupled with electroantennographic detection (GC-EAD) as per Cossé and Bartelt (2000). EAD recordings were made by severing the head of an adult sawfly at the cervix. The head was placed neck down into a vertical well (3× 3 mm) filled with physiological saline located 2 mm from the end of a Plexiglass stage. Antennae were extended horizontally along two shallow channels that ran from the well to the end of the stage. Antennae extended beyond the edge of the stage. A ring of dental clay (1 mm thickness) was placed around the well and covered with a Plexiglass plate to reduce evaporation. The plate was secured with a screw to ensure a water-tight seal. Distal ends of the antennae were severed at the inter-segmental membrane near the 20th antennal segment. EAD connections were made by inserting the distal ends of each antenna into glass capillary silver microelectrodes filled with physiological saline. The antenna with the recording electrode was positioned directly in the effluent stream coming from the GC, and the antennae with the grounding electrode was positioned out of the effluent stream. Compounds from either males or females that elicited antennal activity were identified subsequently based on comparisons to retention times and mass spectra of authentic standards, or to library spectra (as in the case of common well characterized compounds such as nonanal and decanal).

Synthesis 6,14-Pentadecadienal (**1**), 1,9,15-pentacosatriene (**2**), and 1,9-pentacosadiene (**3**) were synthesized as standards using Wittig condensations as the key step (Fig. 1). Reactions were monitored by GC-MS. Attempts were not made to optimize yields. The reagents were obtained from Aldrich Chemical Co., Milwaukee, WI, USA, and solvents were of HPLC grade.

Five Wittig salts were prepared by refluxing the corresponding alkyl halides with triphenylphosphine in acetonitrile (Sonnet 1974). Three of these (**4**, **5**, and **6**) had simple alkyl groups of 1, 10, and 16 carbons, respectively, and two had functionalized alkyl groups (**9** and **12**). To make **9**, 6-bromo-1-hexanol (**7**) was converted to the tetrahydropyranyl (THP) ether (**8**) (Miyashita et al. 1977), which was then converted to the salt. To make **12**, 1,6-dibromohexane (**10**) was first alkylated with allylmagnesium bromide under catalysis by dilithium tetrachlorocuprate as per Johnson et al. (1994); the Grignard reaction gave only monoalkylation. The resulting 9-bromo-1-nonene (**11**) was then converted to the phosphonium salt. Two aldehyde intermediates also were prepared: Octanedial (**14**) was made by oxidizing 1,8-octanediol (**13**) with pyridinium dichromate (PDC) (Corey and Schmidt 1979), and 6,6-dimethoxyhexanal (**16**) was made from cyclohexene (**15**) by ozonolysis, using the procedure of Claus and Schreiber (1986).

To prepare aldehyde **1**, a mixed Wittig reagent was made from salts **4** and **9** in tetrahydrofuran (THF) by treatment with butyllithium, and then dialdehyde **14** was added. The reaction was run essentially as per Sonnet (1974), but no dimethylsulfoxide (DMSO) was added (which Sonnet 1974, used to increase *Z* selectivity). Products with several chain lengths were created, but these differed widely in GC retention, and the THP ether of 6,14-pentadecadien-1-ol (**17**) was recognized readily from its mass spectrum [diagnostic ions, 308 (M), 222 (M-86), and 85 (intense in THP ethers)]. Both the 6*Z* and 6*E* isomers were formed, but 6*Z* isomer was more abundant and eluted earlier by GC. After purification on silica gel (elution with 5% ether in hexane), the THP ether **17** was converted to the free alcohol **18** (Miyashita et al. 1977), and the alcohol was then oxidized with PDC to aldehyde **1**.

When it became clear that **1** was identical to the key compound from female PFW, the synthesis was repeated by a more efficient route (Fig. 1, bottom). A Wittig reaction involving salt **12** and acetal aldehyde **16** (Sonnet 1974) gave just one product chain length, and predominantly the *Z* isomer (82%). After purification of acetal **19** on silica gel (elution with 10% ether in hexane) and by Kugelrohr distillation, **19** was readily hydrolyzed to aldehyde **1** (Ellison et al. 1975). The final product (140 mg, 70% of the theoretical yield) was diluted with hexane and stored at −20°C until needed for field tests.

Standard hydrocarbon triene **2** was prepared in one step by a Wittig reaction involving the decyl salt (**5**) and aldehyde **1**. Standard hydrocarbon diene **3** was made with a

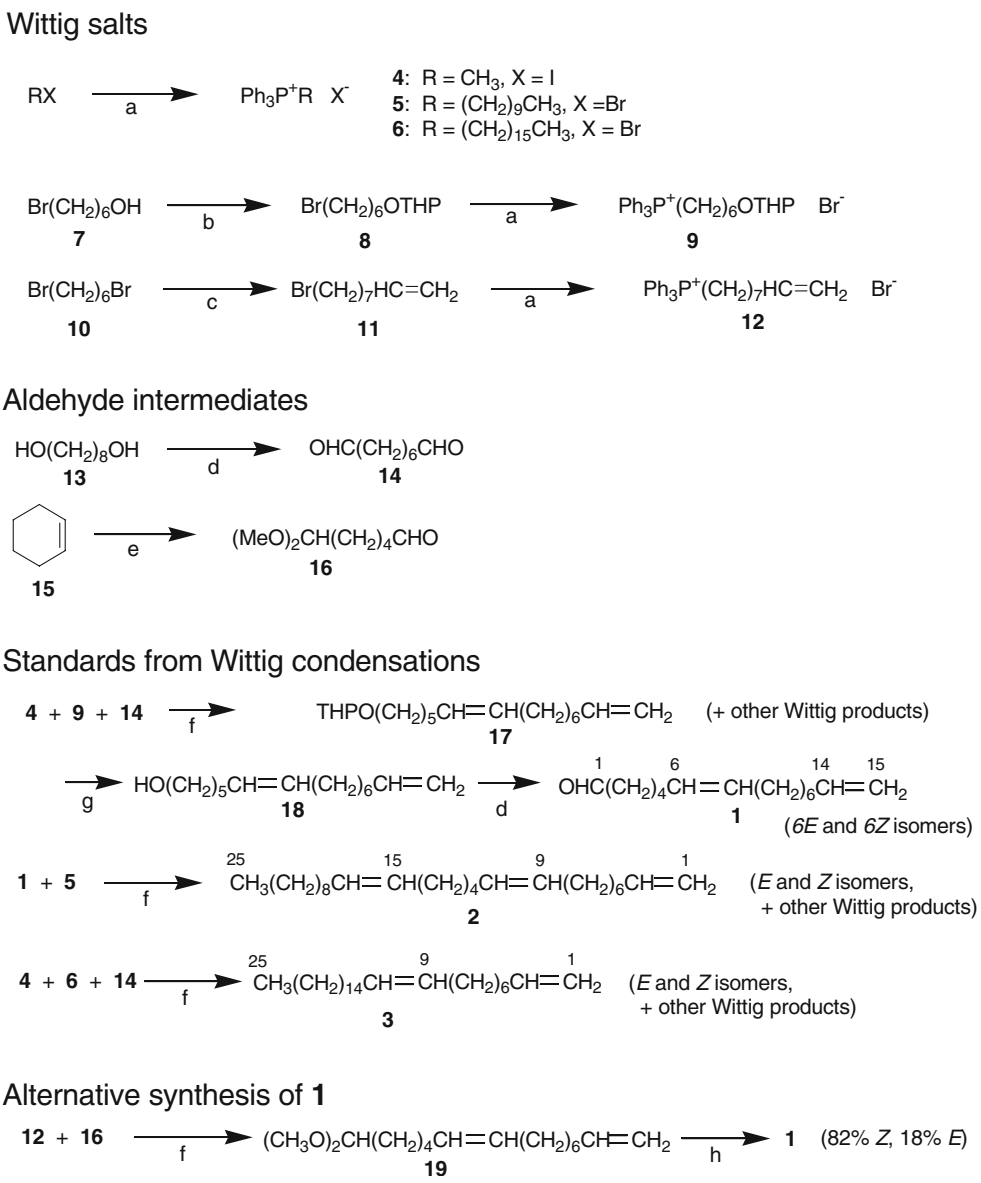

Fig. 1 Synthesis of 6,14-pentadecadienal (**1**), 1,9,15-pentacosatriene (**2**), and 1,9-pentacosadiene (**3**). Reaction conditions: **a** Ph_3P, CH_3CN, reflux, 10 h; **b** 2*H*-3,4-dihydropyran, pyridinium *p*-toluenesulfonate (PPTS), CH_2Cl_2, rt, 4 h; **c** allylmagnesium bromide, Li_2CuCl_4, ether, 0°C, 3 h; **d** pyridinium dichromate, CH_2Cl_2, rt, 4 h; (e) (*i*) O_3, MeOH, CH_2Cl_2, −78°C, until excess O_3 present, purge with N_2, (*ii*) TsOH, −78°C to rt, 1.5 h, (*iii*) $NaHCO_3$, rt, 15 min, (*iv*) Me_2S, rt, 12 h; (f) add BuLi to salt in THF, then add aldehyde, 0°C, 1 h; (g) PPTS, EtOH, 55°C, 2.5 h; (h) 1:2 50% aqueous CF_3COOH:$CHCl_3$, 0°C, 6.5 h

mixed Wittig reaction involving the methyl and hexadecyl salts (**4** and **6**) and dialdehyde **14**. In both reactions, the *Z* isomers predominated and eluted slightly earlier than the *E* isomers on the DB-1 GC column. The hydrocarbons were purified by silica gel chromatography (elution with hexane).

Weathering Experiment To determine if (*Z,Z*)-1-9,15-pentacosotriene could in fact oxidize to produce (*Z*)-6,14-pentadecadienal in the presence of air and ultraviolet light, a 5 μg sample of (*Z,Z*)-1,9,15-pentacosotriene in 100 μl of hexane was applied to the inside wall of a quartz cuvette (Beckman, CA, USA) and the solvent allowed to evaporate, leaving the hydrocarbon as a thin oily film. The cuvette was capped and placed inside a Suntest CPS solar simulator (Atlas, Gainesville, FL, USA), set up as described in McGuire et al. (2000), and exposed to simulated solar radiation for 4.5 h. The cuvette then was rinsed with 500 μl of hexane, and the solution was concentrated to 100 μl and analyzed by using GC-MS. Relative peak area of (*Z*)-6,14-pentadecadienal to (*Z,Z*)-1-9,15-pentacosotriene was determined.

Field Experiments Field testing for male *A. erythrocephala* response to synthetic and natural chemicals was carried out in the spring of 2002 and 2003 at two sites located in central Illinois and upstate New York. Both sites contain persistent outbreaks of *A. erythrocephala* and are remarkably similar in geology, topography, and host species composition, characterized by mixed and monoculture stands of white, red, and Scots pine.

The Illinois site is located in the Sand Ridge State Forest Reserve in Mason County, 50 km southwest of Peoria, Illinois. The reserve encompasses 2,913 ha, made up of

1,584 ha of mixed native oak (*Quercus* sp.) and hickory (*Carya* sp.) forests and 321 ha of fields and sand prairie. The remaining 1,008 ha consists of monoculture and mixed stands of white pine, red pine, and Scots pine. Although local conservation personnel noted feeding damage on pine species at this site for several years (personal communication), the specific defoliator responsible for the damage was not identified until the fall of 2001. This site continues to be infested as of this writing.

The New York field site is located 16 km southeast of Potsdam, New York, at High Flats in Saint Lawrence County. Species composition, geology, and topography of the area are described by Asaro and Allen (1999). An outbreak of *A. erythrocephala* has persisted at this site since the late 1980's and at its peak in the late 1990's covered more than 200,000 ha of mixed forest throughout St. Lawrence and Franklin Counties, New York (Asaro and Allen 1999, 2001). This area continues to be infested, though densities of *A. erythrocephala* appear to have declined in recent years.

Illinois Field Experiments 2002 The first field test of (*Z*)-6,14-pentadecadienal was carried out at the Illinois site from April 17 through April 20, 2002. Treatments included individual rubber septa infused with 50 μg of (*Z*)-6,14-pentadecadienal, 1 mg of nonanal, or 1 mg of decanal, whole body extracts of virgin male or female sawflies, and blank traps which served as controls.

Treatments containing nonanal and decanal were included in the 2002 field tests because these compounds elicit strong antennal activity in both sexes and are also found in aerations and extracts from both sexes. Furthermore, we have observed that decanal can elicit characteristic courtship behaviors in males that includes abdominal flexing and substrate vibration.

Chemical treatments were prepared 24 h prior to field testing by applying 50 μg of (*Z*)-6,14-pentadecadienal, 1 mg of nonanal, or 1 mg decanal each to individual rubber septa (Fisher Scientific, Pittsburgh, PA, USA). Treated septa were stored at −20°C and transported to the field site in Styrofoam coolers containing dry ice. At the field site, treated septa were attached to metal paperclips and hung inside wing traps (Trécé model-1C Great Lakes IPM, Inc., Vestaburg, MI, USA) in single-, double-, or four-compound combinations.

Whole-body extracts of unmated sawflies used in the Illinois field experiments were prepared by collecting pre-emergent adults from the field and extracting groups of either 400 male or 400 female sawflies in 300 ml of hexane for 20 min at room temperature. This method produced approximately 200 ml of extract. Twenty-ml aliquots of extract (roughly 40 insect equivalents) then were poured into plastic Petri dishes (35×10 mm) and allowed to evaporate at room temperature for 1 h, leaving behind an oily residue inside the dish. Petri dishes with residue were capped and sealed with Teflon tape and stored at −20°C before being transported to the field 12 h later in coolers containing dry ice. At the field site, dishes were uncapped and placed in the bottom center panel of individual wing traps.

Treatments used in all field experiments were set out following a randomized design with ten replicates per treatment. Treatments were placed inside wing-traps and secured to wooden stakes 30 cm above ground. At the end of each day, the bottom panel of each trap was removed, and sawflies were tallied per trap. Total trap data from the entire field season were transformed using sqrt (y+1) to meet assumptions of homoscedasticity (Bartlet's test, a=0.05) before performing one-way analysis of variance (ANOVA). *Post hoc* comparisons of the mean number of males caught among treatments were conducted with Tukey's honest significant difference test (Tukey HSD) (α=0.05) using Statistica version 6.0 (StatSoft Inc. Tulsa, OK, USA 2001).

New York Field Experiments 2002 A second field test was conducted at the New York site from May 21 through May 24, 2002. The goal of the second field test was to verify that the synthetic pheromone was indeed active in a different population of *A. erythrocephala*. Treatments at the New York field site included 500 μg and 50 μg doses of (*Z*)-6,14-pentadecadienal, whole body extracts, live virgin male and female sawflies, and blank traps. Because nonanal and decanal did not appear to influence male trap catches in the 2002 Illinois field experiment, they were excluded from the New York field experiments.

Whole-body extracts from virgin male or female sawflies (roughly 40 insect equivalents), were prepared in Illinois, as previously described, 72 h prior to conducting the New York field tests. Bulk extracts were transported to the New York site in sealed 500 liter Erlenmeyer flasks fitted with a rubber stopper sealed with Teflon tape and placed in individual Styrofoam coolers surround by dry ice. Petri dishes containing extracted residue were prepared indoors near the New York field site as described above for the Illinois experiments.

Live virgin male and female sawflies were obtained on site by digging in the ground for pre-emergent adults. Male and female adults also were collected on site and kept in individual translucent containers for 8–18 h prior to placing them in metal screen cylinders (3×5 cm), sealed with cork stoppers. Screen cylinders containing sawflies were hung in the center of each trap with a metal paperclip. Sawflies were replaced every morning. Because data did not adequately meet assumptions of normality or heteroscedasity, differences were analyzed via nonparametric Kruskal-Wallis analysis of variance. *Post-hoc* comparisons were

conducted via multiple comparisons of mean ranks for all pairs of groups (Siegel and Castellan 1988) via Statistica version 6.0 (StatSoft Inc., Tulsa, OK, USA 2001).

Illinois Field Experiments 2003 A third field test was carried out at the Illinois site in 2003 from April 24–27. Treatments included 1.5 μg, 5 μg, 15 μg, 50 μg, 150 μg, and 500 μg doses of synthetic (*Z*)-6,14-pentadecadienal, whole body extracts of virgin male or female sawflies, and live virgin male or female sawflies held in metal cylindrical cages as describe above. Blank wing traps served as controls.

Whole body extracts used in the 2003 Illinois field experiment were prepared as described above for the 2002 field experiments 6 h prior to placing them in the field. In 2003, Petri dishes containing whole body extracts were uncovered and placed in direct sunlight (~21°C air temperature) for 1 h prior to placing them in individual traps. The purpose of exposing whole body extracts to direct sunlight was to determine if exposure to sunlight would enhance the attractiveness of the extract relative to the previous year's results.

Adult male and female sawflies used in the 2003 field experiments were collected as eonymphs and reared at Illinois State University. Adults were kept in translucent 26×39×23 cm plastic containers with wire-screen covers. Containers with fresh sawflies were transported to the field every morning and placed in direct sunlight at approximately 20°C for 1 h prior to placing individual sawflies in screen cages hung centrally inside each trap. As with the whole body extracts, the purpose of exposing live extracts to direct sunlight was to assess whether exposing live female insects would enhance attractiveness. Cages were replaced with fresh sawflies every morning for the duration of the experiment. Total trap data from the entire field season were transformed using log (y+1) to meet assumptions of homoscedasticity (Bartlet's test, a=0.05) before performing one-way analysis of variance (ANOVA). *Post hoc* comparisons were conducted as described above.

Results

Chemical Analysis GC-MS analysis of aerations and whole body extracts obtained from both sexes of *Acantholyda erythrocephala* primarily contained saturated and unsaturated straight chain hydrocarbons, many with a double bond located at the Δ9 position. Qualitative and quantitative differences between sexes included a 25-carbon triene (MW 346), exclusive to females, and a 25-carbon diene (MW 348) that was abundant in females, but also occurred in trace amounts in aerations and extracts obtained from males. A 25-carbon alkene (MW 350) and pentacosane (MW 352) were present in both male and female aerations and extracts (Table 1).

The putative pentacosane was verified based on comparisons to library spectra, GC retention time, and mass spectra of authentic standard (Aldrich Chemical Co., Milwaukee, WI, USA). Preliminary identifications of the -ene, -dien, and -trien, were based on GC-MS analysis of their DMDS derivatives.

DMDS adducts of pentacosene (Fig. 2a) gave a molecular ion at *m/z* 444 (350+94), consistent with a 25-carbon chain containing two thiomethyl (CH_3S) groups. Diagnostic fragments A^+ (*m/z* 173) and B^+ (*m/z* 271) indicated a double bond at the 9 position (9-pentacosene).

DMDS adducts for pentacosadiene (Fig. 2b) produced a molecular ion at *m/z* 536 (348 + 188). This was consistent with a 25-carbon chain containing four CH_3S groups. Key diagnostic ions B^+ (*m/z* 265, 217,169) and D^+ (*m/z* 271) also suggested a double bond at the 9 position. Ion fragments A^+ (*m/z* 61) and C^+ (*m/z* 441, 393, 345) indicated the presence of a terminal double bond, resulting in 1,9-pentacosadiene.

DMDS adducts for pentacosatriene (Fig. 2c) produced a molecular ion at *m/z* 628 (346 + 282), consistent with a 25-carbon chain containing six CH_3S groups. Ion fragments A^+ (*m/z* 61) and D^+ (m/z 567, 519, 471), again indicated a terminal double bond. Fragments B^+ (*m/z* 265, 217, 169) and E^+ (383, 315, 267) indicated a double bond at the 9 position. Fragments C^+ (441, 393, 345) and F^+ (187) suggested a third double bond at the 15 position (1,9,15-pentacosatriene).

Synthetic hydrocarbons corroborated these identifications and helped clarify double bond configurations. Female-derived 1,9-pentacosadiene and 1,9,15-pentacosatriene showed GC retention times and mass spectra identical to the earliest-eluting (*Z*) synthetic isomers indicating that these hydrocarbons existed in the *Z* configuration (i.e., (*Z*)-1,9-pentacosadiene and (*Z,Z*)-1,9,15-pentacosatriene). DMDS derivatives of synthetic 1,9-pentacosadiene and 1,9,15-pentacosatriene produced mass spectra and GC retention times identical to DMDS derivatives of the natural 1,9-pentacosadiene and 1,9,15-pentacosatriene.

GC-MS analysis of natural and DMDS derivatized whole body extracts from ten male and ten female sawflies in hexane revealed that female sawflies contained a number of dienes with double bonds at the Δ1 and Δ9 positions. These dienes were either nonexistent in male extracts or present only in trace amounts (Table 1).

Electrophysiology Electrophysiological analysis of male and female aerations showed antennal sensitivity in both sexes to a series of aldehydes including nonanal, decanal, and possibly 2-undecanal. Male-specific antennal responses were also recorded for a single unknown compound (Fig. 3)

Table 1 Putative identification of major cuticular hydrocarbons collected from whole body extracts of ten male and ten female *Acantholyda erythrocephala* (4 hrs in hexane)

Name	ECL	Relative amount (% of total peak area)		MS Data: Molecular Ion and diagnostic ions. (and key ions adducts DMDS adducts)	
		Females	Males		
n-Alkanes					
Heneicosane	21.00	7.9	5.3	296	
Docosane	22.00	0.9	0.6	310	
Tricosane	23.00	17.2	14.4	324	
Tetracosane	24.00	0.4	0.3	338	
Pentacosane	25.00	7.3	4.6	352	
Hexacosane	26.00	0.9	2.2	366	
Heptacosane	27.00	12.4	8.9	380	
n-Alkenes					
9-tricosene	22.70	0.5	0.5	322	(173, 243, 416)
7-tricosene	22.77	0.4	0.3	322	(145, 271, 416)
1-tricosene	22.96	0.5	0.5	322	(61, 369, 416)
9-tetracosene	23.74	0.3	1.7	336	(173, 257, 430)
9-pentacosene	24.75	7.9	55	350	(173, 271, 444)
1-pentacosene	24.97	0.3	0.1	350	(61, 383, 444)
9-heptacosene	26.61	1.1	0.2	378	(173, 299, 472)
7-heptacosene	26.82	0.3	4.9	378	(145, 327, 472)
n-Alkadienes					
1,9-tricosadiene	22.61	0.2	nd	320	(61, 169, 217, 243, 265, 351, 399, 447, 508)
1,9-tetracosadiene	23.65	1.1	nd	334	(61, 169, 217, 265, 257, 379, 427, 475, 522)
1,9-pentacosadiene	24.70	37.7	tr	348	(61, 169, 217, 265, 271, 379, 422, 475, 536)
1,9-heptacosadiene	26.54	1.5	nd	376	(61, 169, 217, 265, 299, 407, 455, 503, 564)
n-Alkatrienes					
1,9,15-pentacosatriene	24.35	0.5	nd	346	(61, 169, 187, 217, 265, 267, 315, 345, 393, 441, 471, 519, 567, 628)
heptacosatriene	26.23	0.1	nd	374*	
Methyl Substituted Alkane					
3-methyltricosane	23.79	0.6	0.5	281, 309, 338	

ECL = equivalent chain length, *nd* = not detected in whole body extracts, *tr* = trace amounts detected (<1% of major isomer)

*ID based on retention time and mass spectra only

isolated exclusively from moderately polar fractions (10% ether) of female aerations and whole body extracts (Fig. 4). Key diagnostic ions obtained from GC-MS analysis of female aerations were *m/z* 222 (M), 204 (M-18), and were of low intensity (3.1% and 2.9%, respectively, of base peak) (Fig. 5). The apparent molecular weight of 222, likely loss of water (*m/z* 204), and the modest polarity on silica suggested a 15-carbon aldehyde with two double bonds. However, the amount of material obtained from the natural extracts was too small for double bond locations to be determined.

Based on previous reports from two other species of sawfly (Bartelt and Jones 1983; Bartelt et al. 1983, 2002; Cossé et al. 2002), and one Braconid species (Swedenborg and Jones 1992) suggesting that aldehyde pheromones could result from natural oxidation of unsaturated cuticular hydrocarbon precursors, we considered all potential oxidation products of (*Z,Z*)-1,9,15-pentacosatriene, a compound that was found exclusively in aerations and whole body washes of female *A. erythrocephala*, (see Frankel 1998 for a review of chemical mechanisms for natural oxidation of unsaturated lipids). We predicted that oxidation of (*Z,Z*)-1,9,15-pentacosatriene could yield a 15-carbon aldehyde containing two double bonds at the six and 14 positions. GC retention time, mass spectrum, and male-specific antennal activity of synthetic (*Z*)-6,14-pentadecadienal was found to be identical to the 15-carbon aldehyde also obtained exclusively from aerations and whole body washes of female *A. erythrocephala* (Figs. 4 and 6). Female sawflies showed no antennal sensitivity to natural or

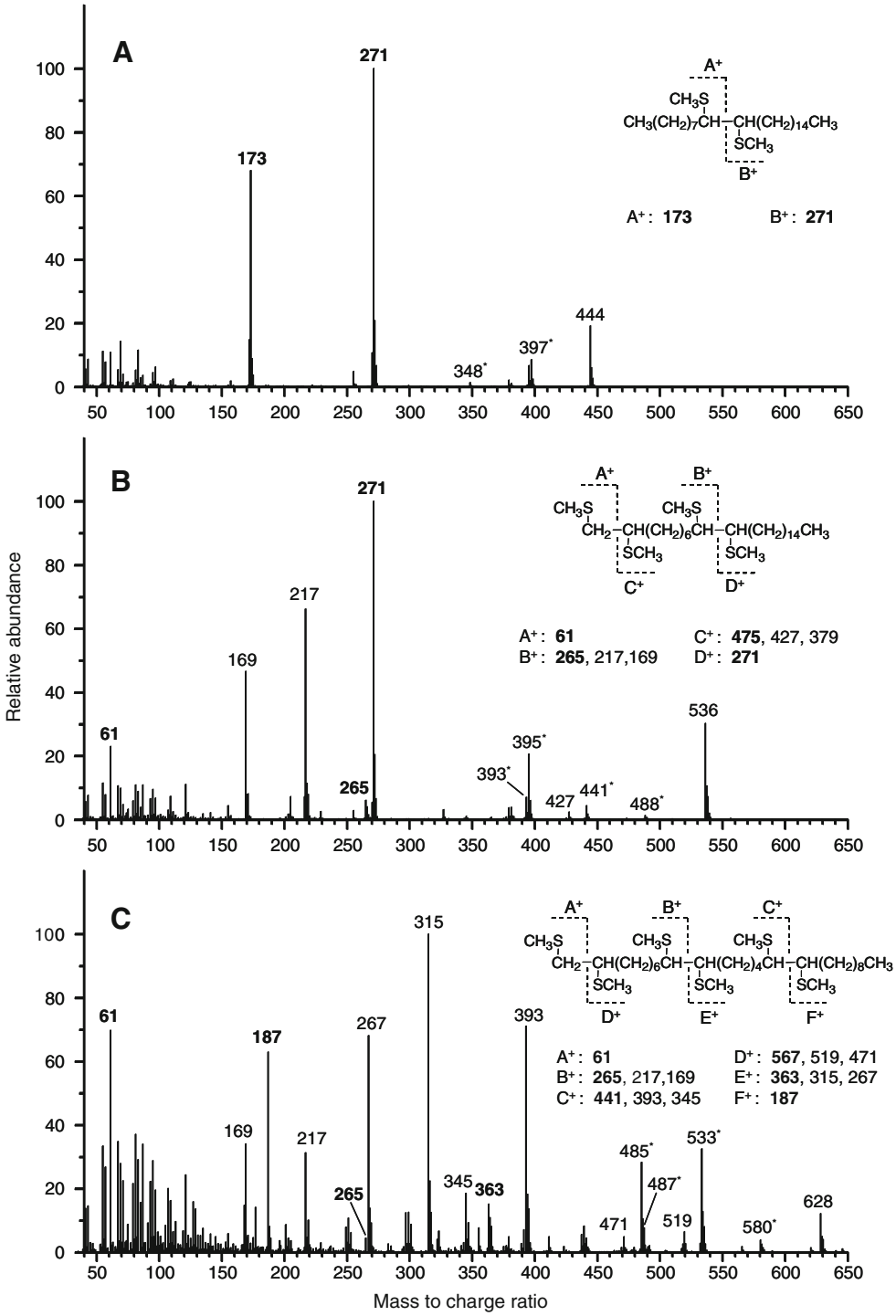

Fig. 2 Mass spectra of DMDS adducts from female-derived 9-pentacosene (Trace A), 1,9-pentacosadiene (Trace B), and 1,9,15-pentacosatriene (Trace C), and explanation of fragmentation patterns. Key one-bond cleavage fragments shown in bold type (some have very low intensity), followed by fragments representing elimination of one or two CH_3SH groups (48 mass units). Eliminations of one or more CH_3S- or CH_3SH from the molecular ion indicated in the mass spectra by (*)

synthetic (*Z*)-6,14-pentadecadienal (Fig. 6) suggesting that this compound had a sex-specific role in the mating system of *A. erythrocephala*.

Weathering Experiment After 4.5 h of exposure to simulated solar radiation, approximately 1% of (*Z,Z*)-1,9,15-pentacosatriene was oxidized to (*Z*)-6,14-pentadecadienal thereby providing a plausible mechanism for pheromone production in this insect.

Illinois Field Experiments, 2002 Data obtained from the 2002 Illinois Field Experiments showed that traps baited with (*Z*)-6,14-pentadecadienal, alone or in combination with nonanal and/or decanal, caught significantly greater numbers of males relative to all other treatments and blank controls (ANOVA, $F=10.83$; $df=9, 90$; $P<0.01$) (Fig. 7). There were no differences in the number of males caught in traps baited with nonanal or decanal, singly or in combination, relative to whole body washes from either

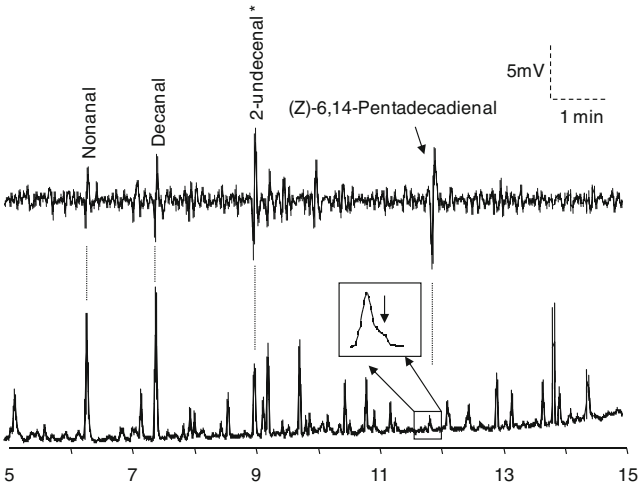

Fig. 3 Male antennal response (*top trace*) and GC detector response (*bottom trace*) to moderately polar silica fraction (10% either in hexane) obtained from female whole body extract of virgin females. Antennally active peaks, from *left* to *right*, correspond to nonanal, decanal, 2-undecenal (identification based on analysis of GC-retention time and mass spectra), and (*Z*)-6,14-pentadecadienal

sex (40 insect equivalents per trap) or blank controls (Tukey HSD, $P>0.05$). The addition of lures containing synthetic nonanal and/or decanal to traps baited with (*Z*)-6,14-pentdecadienal did not significantly improve field attraction relative to traps containing (*Z*)-6,14-pentdecadienal alone. Although there was a slightly greater number of males caught in traps baited with whole body extracts of virgin females relative to blank controls, these results were not significantly different from blank controls (Tukey HSD, $P>0.05$).

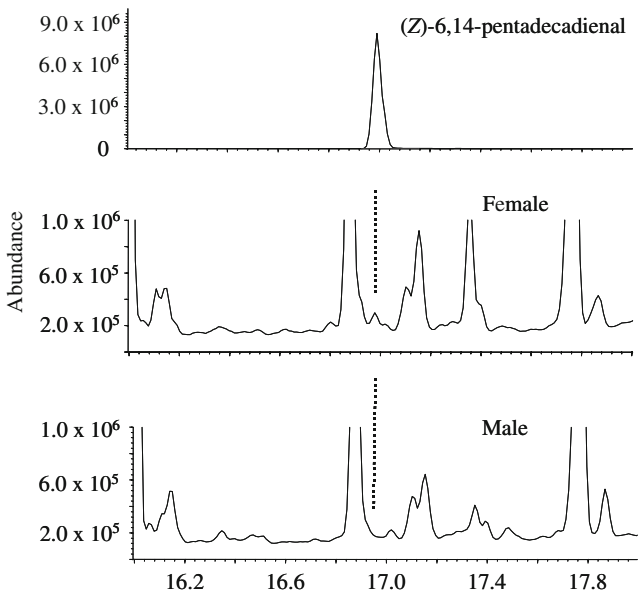

Fig. 4 Total ion chromatographs for synthetic (*Z*)-6,14-pentadecadienal (1 ng) (*top trace*), fractions (10% either in hexane) of whole body extract obtained from a single female (*middle trace*), and a single male (*bottom trace*) *A. erythrocephala*

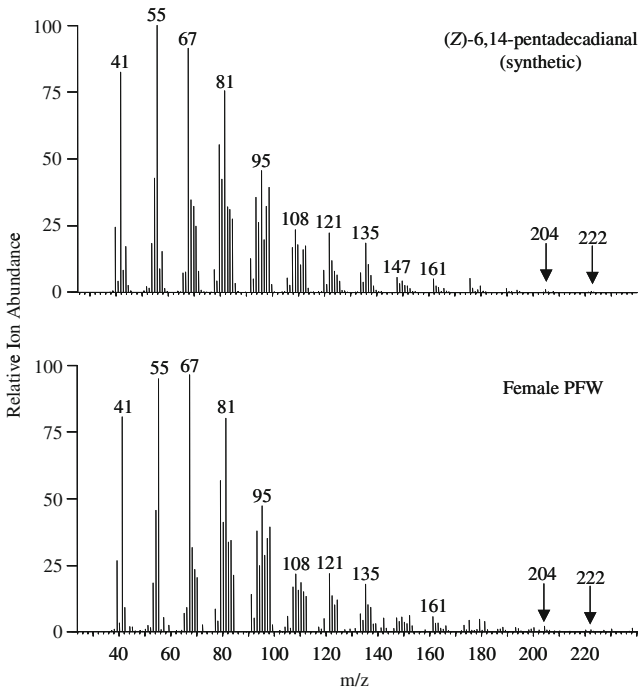

Fig. 5 Mass spectra of synthetic (*top trace*) and naturally occurring (*bottom trace*) (*Z*)-6,14-pentadecadienal in female *Acantholyda erythrocephala*

New York Field Experiments, 2002 Traps baited with 500 μg and 50 μg concentrations of (*Z*)-6,14-pentadecadienal caught significantly more males than all other treatments (Kruskal-Wallis ANOVA by Ranks, H=38.13 ($N_{treatments}=6$, $N_{total\ observations}=65$), $P<0.01$) (Fig. 8). *Post-hoc* comparisons were carried out via multiple comparisons of *z* values for mean ranks among pairs of treatments. There was no significant difference in the number of males caught in traps baited with 500 μg and 50 μg of (*Z*)-6,14-pentadecadienaln nor were there significant differences between traps baited with live sawflies or

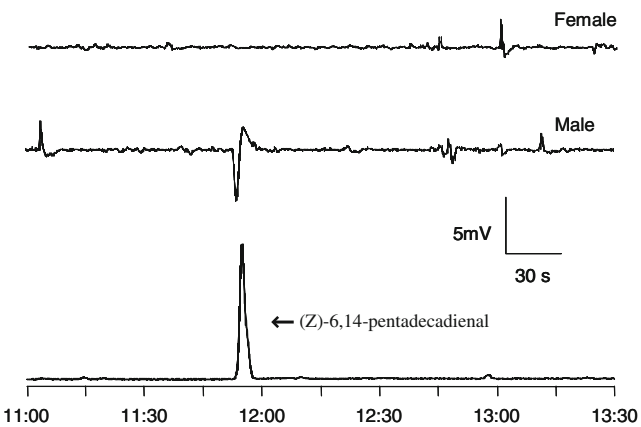

Fig. 6 GC-EAD trace showing antennal responses by female (*top trace*) and male (*middle trace*) *Acantholyda erythrocephala* to synthetic (*Z*)-6,14-pentadecadienal (GC detector trace at *bottom*)

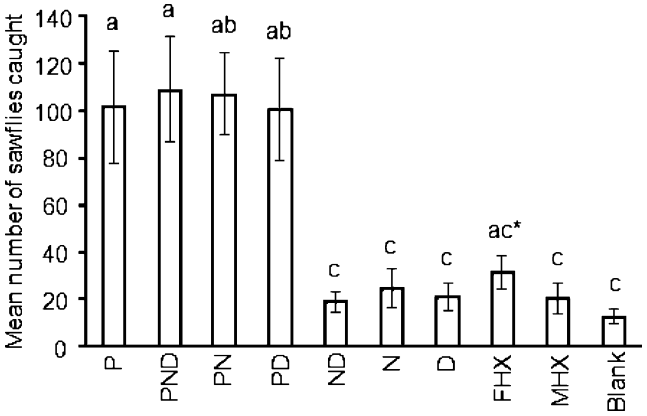

Fig. 7 Illinois field experiment 2002. Results showing the mean number of male *Acantholyda erythrocephala* collected per treatment from April 17 through April 20. Error bars = standard error of untransformed data. Different letters indicate significant differences. Treatments include *P*=(*Z*)-6,14-pentdecadienal (50 μg), *N*=nonanal (1 mg), D = decanal (1 mg), ND = nonanal and decanal (1 mg each), FHX = female whole body washes (in hexane), MHX = male whole body washes (in hexane), and blank traps

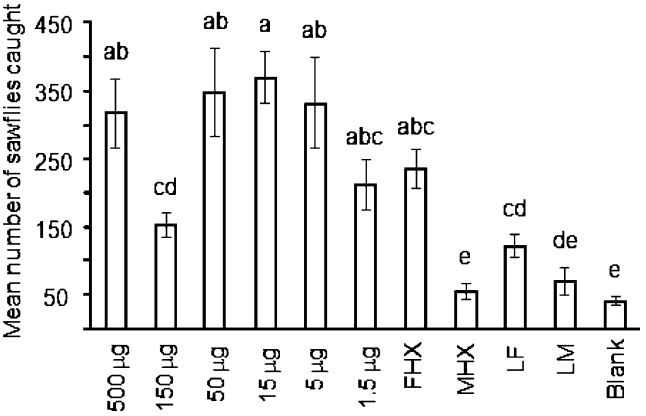

Fig. 9 *Illinois field experiment 2003*. Mean number of sawflies collected from April 24–27. Treatments include 500 μg; 150 μg; 50 μg; 15 μg; 5 μg; and 1.5 μg of (*Z*)-6,14-pentdecadienal. Error bars = SE. Other treatments include, FHX = female whole body washes (in hexane); LF = live females; MHX = male whole body washes (in hexane); LM = live males; and blank traps. Different letters designate significant differences

whole body washes relative to blank controls (multiple comparisons of *z* values for mean ranks $P>0.05$) (Fig. 8). Note that some treatments were lost due to animal damage.

Illinois Field Experiments, 2003 Significantly larger number of males were caught in traps baited with synthetic (*Z*)-6,14-pentadecadienal as well as female whole body washes compared with live males, male whole body washes, and blank controls (ANOVA $F=21.65$; $df=10, 99$; $P<0.01$) (Fig. 9). Traps containing whole body extracts of live virgin females attracted significantly more (Tukey HSD, $P<0.05$) males than those baited with male extracts or blank controls, but were not significantly different from traps baited with live virgin males (Tukey HSD, $P>0.05$).

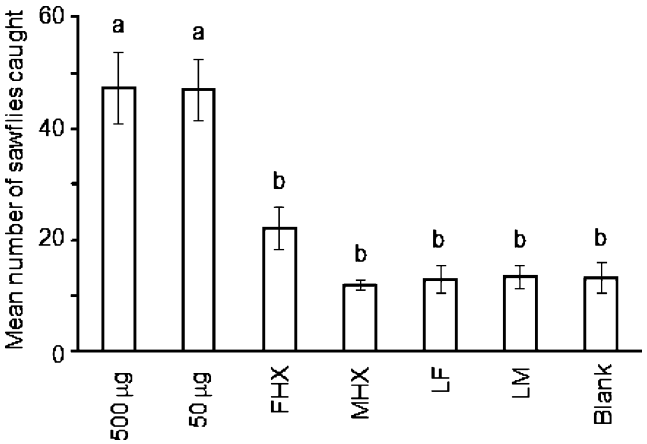

Fig. 8 New York field experiment 2002. Results showing the mean number of male *Acantholyda erythrocephala* collected per treatment from May 21 through May 24. Error bars = SE of untransformed data. Different letters indicate significant differences. Treatments include 500 μg [(*Z*)-6,14-pentdecadienal] (*N*=9); 50 μg [(*Z*)-6,14-pentdecadienal] (*N*=10); FHX = female whole body washes (in hexane) (*N*=9); LF=live females (*N*=8); MHX=male whole body washes (in hexane) (*N*=10); LM = live males (*N*=10); and blank traps (*N*=9)

Discussion

In this paper, we provide laboratory and field results demonstrating that (*Z*)-6,14-pentadecadienal, a compound derived exclusively from a female *A. erythrocephala*, functions as a attractant pheromone for male conspecifics. This is the first pheromone identified for the genus *Acantholyda* and the second to show pheromone-like properties in the family Pamphiliidae.

GC-MS analysis of aerations and whole-body extracts revealed that cuticular hydrocarbons from male and female sawflies were made-up primarily of unbranched straight chain hydrocarbons with little evidence of methyl branching. Major differences observed in cuticular hydrocarbons collected from male and female sawflies, included (*Z,Z*)-1,9,15-pentacosatriene, found exclusively in female aerations and whole body extracts, and a number of 1,9-dienes that were either more abundant or exclusive to female aerations and whole-body extracts (Table 1).

By considering potential oxidation products of (*Z,Z*)-1,9,15-pentacosatriene, we were able to gain valuable insight regarding the final chemical structure for (*Z*)-6,14-pentadecadienal. We also were able to demonstrate that production of (*Z*)-6,14-pentadecadienal can occur via abiotic oxidization of (*Z,Z*)-1,9,15-pentacosatriene. Although these results illustatrate a plausible mechanism for pheromone production in this species, they do not exclude the possibility that (*Z*)-6,14-pentadecadienal also may be produced through some dedicated biosynthetic pathway.

Field results from the Illinois and New York sites in 2002 and 2003 demonstrate that (*Z*)-6,14-pentadecadienal functions as an effective male lure. It is also interesting to note that in the 2003 Illinois field experiments, significantly more male sawflies were captured in traps baited with whole body extracts of virgin females relative to traps baited with extracts of virgin males after they were exposed to direct sunlight (Fig. 9). These observations fit well with laboratory experiments that demonstrate that (*Z*)-6,14-pentadecadienal can indeed arise via abiotic oxidation of female specific (*Z,Z*)-1,9,15-pentacosatriene.

Although we recorded antennal responses to nonanal and decanal in both male and female sawflies, neither of these compounds was found to significantly influence male attraction in the field. Similarly, neither compound exhibited synergistic or inhibitory influences on male response to (*Z*)-6,14-pentadecadienal. Nevertheless, these results do not rule out a bioactive role for these compounds. Indeed, it is interesting to note that *n*-decanal and 2-undecenal, respectively, not only stimulate courtship behavior and antennal responses in male *A. erythrocephala*, but both compounds also are likely oxidation products of (*Z*,*Z*)-1,9,15-pentacosatriene via either α-scission or β-scission of the C15 double bond. Future planned research will explore the role of 2-undecenal in the behavior of *A. erythrocephala*.

To date, approximately 22 pheromones have been identified among 14 families and 8,000 species (Smith 1993) of sawfly. Most of these pheromones have been reported in the Diprionidae and are made up of various methyl branched acetate or propionate products of diprionol (Jewett et al. 1976; Ahlgren et al. 1979; Anderbrant et al. 1992, 2000, 2005; Bergström et al. 1995, 1998; Chen et al. 1997; Tai et al. 1998, 2002; Wassgren et al. 2000; Ostrand et al. 2003; Hedenström et al. 2006). *Acantholyda erythrocephala* differs from diprionids in that its pheromone, (*Z*)-6,14-pentadecadienal, and likely pheromone precursor, (*Z,Z*)-1,9,15-pentacosatriene, are both simple unbranched straight chain hydrocarbons. Although this simple straight chain structure differs in comparison to active pheromones of diprionind species, it is not surprising in terms of a cuticular precursor or as a pheromone. Indeed, straight chain cuticular hydrocarbons, including those with a double bond in the Δ-9 position, are quite common among insect cuticular hydrocarbons (Blomquist et al. 1993; Howard 1993; Howard and Blomquist 2005; Steiner et al. 2007). Nevertheless, pheromone production by means of abiotic oxidation remains a relatively little explored area of study in insect chemical ecology. Other reports describing pheromone production by means of abiotic oxidation in insects include the yellow-headed spruce sawfly, *Pikonema alaskensis*, (P.) (Tenthredinidae) (Bartelt and Jones 1983), the parasitic wasp of the European corn borer *Ostrinia nubilalis* Hubner, *Macrocentrus grandii* Goidanich (Hymenoptera: Braconidae) (Swedenborg and Jones 1992), and the wheat stem sawfly, *Cephus cinctus* Norton (Hymenoptera: Cephidae) (Bartelt et al. 2002; Cossé et al. 2002). The results presented here, therefore, represent only the fourth report of pheromone production in an insect species that uses this mechanism.

With the development of a viable pheromone lure for *A. erythrocephala*, we now offer an additional management tool for monitoring and possibly controlling this forest pest.

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aversion; and 4) flavor modification. Known repellent formulations employ these mechanisms singly or in combination.

At the most basic level, all repellents may deter herbivores by exploiting their fear of unfamiliar visual, olfactory, or taste cues (neophobia). However, repellents that rely on neophobia alone (there are no additional negative consequences associated with them) are subject to habituation and will not be avoided for extended periods (Nolte 1999). Visual and vapor repellents often rely on neophobia. Repellent stimuli disassociated from the food source (not applied directly to the food) can be referred to as "vapor repellents" as they are not ingested (thus, do not contribute to the flavor of the repellent stimuli) and are detectable at variable distances from the source.

Most contact repellents (applied directly to the plant) employ active ingredients that impart additional consequences beyond neophobia. One such mechanism is associated with activation of the trigeminal system. The consequence of peripheral (oral/nasal/ocular) contact with these repellents is pain. Among mammals, capsaicin is a well-known trigeminal irritant (Nolte and Wagner 2000). Another consequence of ingesting certain repellents is malaise. Repellent compounds that produce negative postingestive consequences (i.e., malaise or gastrointestinal distress) are avoided as a result of learning. This mechanism is often termed aversion learning or conditioned aversion (Burritt and Provenza 1989). The active ingredient required to produce the negative consequences is typically a toxin. Sensory cues of the repellent formulation (usually flavor) are associated with the negative consequences of toxin ingestion and are avoided at future encounters. In laboratory studies, lithium chloride often is used as the toxin to condition aversions (Riley and Tuck 1985). In formulated repellents, thiram (tetramethylthiuram disulfide) is a fungicide used to condition aversions (Nolte and Wagner 2000). Among other symptoms, chronic thiram exposure produces anemia and nausea (Maita et al. 1991).

Numerous compounds have been used to alter the flavor of treated plants without eliciting pain or malaise. One such strategy has been to employ compounds that impart bitter taste. In practice, repellents employing only bitter compounds are typically ineffective as deer repellents (Nolte and Wagner 2000) and there is some question about the reliability of bitter taste *per se* as a warning of toxicity (Glendinning 1994; Nolte et al. 1994b). Blood and egg are examples of ingredients that yield effective herbivore repellency when applied to plants without causing pain or malaise (Nolte and Wagner 2000). In recent years, hydrolyzed casein (HC) has been added to the list of stimuli that produce long-lived avoidance (Kimball and Nolte 2006). Repellent ingredients like blood, egg, and HC are non-toxic (i.e., unlikely to condition aversions) and typically are not subject to habituation in repeated tests (i.e., unlikely to cause avoidance merely via neophobia).

The relative effectiveness of repellents that rely on any of these mechanisms may depend on the individual herbivore's motivation to consume the protected resource. For example, when alternative foods are available, shiny ribbons (a visual repellent with no consequence) may provide significant protection in localized areas. However, when alternative foods are scarce, repellents with actual consequences to the consumer may be required to reduce browsing effectively. Previous studies of herbivore repellents failed to account for feeding motivation. In this study, the incentive to consume test diets was manipulated by allowing captive deer to learn about two test diets that differed in energy content. A series of experiments then were conducted to compare the different mechanisms of deer repellency and evaluate repellent effectiveness when incentive to consume the treated diet was varied.

Methods and Materials

Subjects Ten hand-reared white-tailed deer (*Odocoileus virginianus*) were group housed in a large (ca 2 ha) outdoor pen except during individual bioassays. Shelter, water, and mineral block were available *ad libitum*. Basal diet was provided at varying intervals: *ad libitum* on days with no scheduled bioassays and overnight from 1600 h to 0800 h daily in advance of individual bioassays. Thus, subjects were restricted from basal diet for 6 h. For individual bioassays, deer were led into individual pens (sheltered stalls measuring approximately 5×3 m). Water was provided in the rear of the stalls, and access doors located at the head of each stall allowed for placement and removal of plastic feed containers (ca 50 cm diam and 15 cm deep). This study was approved by the National Wildlife Research Center's Institutional Animal Care and Use Committee (QA-1642) and conducted during the period of 24 March to 25 April 2009.

Diets Three different pelleted diets were used during the study, including a basal diet familiar to the subjects (Antler Max®; Purina Mills, St. Louis, MO, USA). All test subjects had several years experience with Antler Max® as their primary food source. Two test diets were formulated to differ in net energy while containing similar protein (Table 1; X-Cel Feeds, Tacoma, WA, USA). High energy (HE) and low energy (LE) test diets were distinctly flavored with citrus-anise-vanilla or maple-anise flavors, respectively, to facilitate easy discrimination during bioassays (Table 1). Animals learn about foods they eat by integrating flavor with the postingestive consequences of consuming that food (Provenza 1995a). Preferences (or aversions)

Table 1 Composition and nutritional content of the high energy (HE) and low energy (LE) test diets

Ingredient	High energy (HE)	Low energy (LE)
Barley	22%	15%
Corn grain, ground	35%	7%
Corn, distillers	9%	6%
Wheat mill run	0	15%
Alfalfa meal	6%	19%
Soybean hulls	0	14%
Beet pulp	13%	14%
Soybean meal	11%	7%
Minerals and vitamins	3%	2%
Dry dairy krave® flavor[a]	0.1%	0
Anise-maple flavor	0	0.1%
Crude protein	15.3%	15.3%
Non-structural carbohydrates	47.3%	21.3%
Relative feed value	430	152
Net energy gain	1017 Mcal	803 Mcal

[a] Citrus-anise-vanilla and other natural flavors

based on flavor are formed such that these flavors are recognized readily at future encounters. Upon learning by the subjects, the distinct flavors were expected to be readily associated with the energy content of the food.

Repellents Test diets were treated with commercially-obtained repellents according to labeled use as specified by the manufacturers. Deerbusters® sachets (Trident Enterprises, Frederick, MD, USA) represented the neophobia mechanism. The irritation mechanism was represented by Miller's Hot Sauce® (Miller Chemical and Fertilizer Corp., Hanover, PA, USA). Chew-Nott® (Nott Products, Coram, NY, USA) that contained the fungicide thiram was the repellent chosen for conditioned aversion. The final mechanism, flavor modification, was represented by Plantskydd® (Tree World Plant Care Products Inc., St. Joseph, MO, USA), which contains blood meal.

Sachets similar to those marketed as Deerbusters® repellent, but containing only meat and bone meal were used as a vapor repellent (the repellent was not in contact with the diets). Unlike the usual commercial product, our experimental sachets did not contain capsaicin (irritant). As such, the sachets were suited perfectly for this study because their mode of action was limited to neophobia—largely owing to the fact that the meat and bone meal was not applied directly to the test diets. Sachets were attached to the inside of feed bowls by use of zip-ties passing through two holes drilled near the top edge of the bowl.

Two hundred and forty mL of Miller's Hot Sauce® (2.5% capsaicin) were mixed with 5 mL Tactic® (a latex-based sticker; Loveland Industries, Greeley, CO, USA) and 4.0 L tap water (resulting in a 0.14% capsaicin solution). A hand-held pump sprayer was used to treat test diets until the pellets were visibly coated, and were allowed to dry overnight. Approximately 40 ml were used to treat 2 Kg of diet. Two additional contact repellents were similarly prepared according to label directions and applied directly to the test diets. Chew-Nott® (20% thiram) was mixed 1:1 with tap water prior to application and Plantskydd® was employed as the ready-to-use formulation (Tree World Plant Care Products Inc., St. Joseph, MO, USA) consisting of 16.7% dried porcine and/or bovine blood.

Pre-trial Experience with Test Diets For 2 wk prior to individual bioassays, either HE or LE test diets were provided *ad libitum* in group housing according to a predetermined schedule (Table 2). Pre-trial exposure was designed to promote association of energy content of the diets with their specific flavors. During group feeding, the two test diets were offered in separate 100-L feed bins. For individual bioassays, HE diet always was presented in a blue-colored bowl, and LE diet was presented in a black-colored bowl—regardless of presence or absence of repellent treatment.

Experiment 1: Diet Preference Subjects were led/herded into individual stalls and untreated test diets (HE and LE) were offered in a two-choice test for two consecutive days (days 16 and 17; Table 2). The right/left position of the diets was predetermined and alternated on the 2nd day. The 30 min bioassays commenced at 1400 h daily following a 6 h period of basal-diet restriction. Intake of each diet was determined by difference (mass immediately prior to and after the 30 min bioassay).

Experiment 2: Repellency and Feeding Incentive Experiment 2 was initiated the following day and similarly employed two-choice tests with HE and LE in 30 min trials. The purpose of this experiment was to offer a choice

Table 2 Pelleted diets offered *Ad libitum* to test subjects in group housing before, during, and in between experiments 1, 2, and 3 (HE = High energy diet; LE = Low energy diet; BOTH = both HE and LE; MAX = Antler max®)

		Day1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
		LE	LE	HE	HE	LE
Day 6	**Day 7**	**Day 8**	**Day 9**	**Day 10**	**Day 11**	**Day 12**
LE	HE	LE	HE	LE	HE	LE
Day 13	**Day 14**	**Day 15**	**Day 16** ***Exp. 1***	**Day 17** ***Exp. 1***	**Day 18** ***Exp. 2***	**Day 19** ***Exp. 2***
HE	Both	Both	Max	Max	Max	Max
Day 20 ***Exp. 2***	**Day 21** ***Exp. 2***	**Day 22** ***Exp. 2***	**Day 23** ***Exp. 2***	**Day 24** ***Exp. 2***	**Day 25** ***Exp. 2***	**Day 26**
Max	Max	Max	Max	Max	Max	Both
Day 27	**Day 28** ***Exp. 3***	**Day 29** ***Exp. 3***	**Day 30** ***Exp. 3***	**Day 31** ***Exp. 3***	**Day 32** ***Exp. 3***	**Day 33** ***Exp. 3***
Both	Max	Max	Max	Max	Max	Max

of treated diet and untreated alternative, while also varying the incentive to consume the treated diet. One diet was treated with a single repellent treatment, while the other remained unadulterated (Table 3). For example, one subject was offered a choice of HE diet treated with blood and untreated LE in a two-choice test, while another subject was offered a choice of LE diet treated with blood and untreated HE (i.e., the opposite diet-treatment pair). Each comparison was repeated on consecutive days with the right/left position determined in advance and alternated on the 2nd day. Each subject was tested with all four repellent treatments in four of the eight possible combinations of diet (HE or LE) and repellent in a balanced incomplete block design. As a result, all possible treatment and diet combinations were replicated five times over the 8 d experiment (Table 3). Intake of each diet was determined by difference (pre- and post-bioassay mass).

Experiment 3: Pair-wise Repellent Comparison Following a 2 d intermission, experiment 3 consisted of two-choice tests conducted with HE-treated diets (Table 4). Each diet was treated with a single repellent, and the four different treatments were compared pair-wise such that comparisons were repeated on consecutive days, and each subject was tested with three of the six possible comparisons (Table 5). As a result, all possible pair-wise comparisons were replicated five times over the 6 d experiment. Intake of each diet was determined by difference (pre- and post-bioassay mass).

Statistical Analyses Data from each experiment were analyzed separately. Preference scores (intake of one diet divided by the sum of both diets) from two-choice tests were analyzed by mixed model analyses of variance (ANOVA), and residual plots were generated to evaluate ANOVA assumptions. Outliers (defined as having studentized residuals greater than 3 or less than −3) were removed from the data set prior to all analyses. Subject was a random effect in all models. When necessary, the null hypothesis of indifference (defined as a preference score of 0.5) was tested by using the value 0.5 minus the preference score as the response in the model.

Day, position of the HE diet (right or left), and the interaction (day*position) were fixed effects in experiment 1. Diet preference was first evaluated by examining the distribution of mean HE preference scores (2 d averages for

Table 3 Low energy (LE) and high energy (HE) diets were offered in two-choice tests in experiment 2. One choice was treated with one of the repellent ingredients as indicated in parentheses (S = Sachet; T = Thiram; B = Blood; C = Capsaicin)

Subject	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
5	HE(S) vs. LE	HE(S) vs. LE	LE(T) vs. HE	LE(T) vs. HE	HE(B) vs. LE	HE(B) vs. LE	LE(B) vs. HE	LE(B) vs. HE
21	HE(B) vs. LE	HE(B) vs. LE	LE(C) vs. HE	LE(C) vs. HE	HE(T) vs. LE	HE(T) vs. LE	LE(S) vs. HE	LE(S) vs. HE
22	HE(T) vs. LE	HE(T) vs. LE	LE(B) vs. HE	LE(B) vs. HE	HE(S) vs. LE	HE(S) vs. LE	LE(C) vs. HE	LE(C) vs. HE
25	LE(B) vs. HE	LE(B) vs. HE	HE(S) vs. LE	HE(S) vs. LE	LE(C) vs. HE	LE(C) vs. HE	HE(T) vs. LE	HE(T) vs. LE
92	HE(C) vs. LE	HE(C) vs. LE	LE(S) vs. HE	LE(S) vs. HE	HE(B) vs. LE	HE(B) vs. LE	LE(T) vs. HE	LE(T) vs. HE
93	LE(S) vs. HE	LE(S) vs. HE	HE(C) vs. LE	HE(C) vs. LE	LE(T) vs. HE	LE(T) vs. HE	HE(B) vs. LE	HE(B) vs. LE
95	LE(C) vs. HE	LE(C) vs. HE	HE(T) vs. LE	HE(T) vs. LE	LE(B) vs. HE	LE(B) vs. HE	HE(S) vs. LE	HE(S) vs. LE
97	LE(T) vs. HE	LE(T) vs. HE	HE(B) vs. LE	HE(B) vs. LE	LE(S) vs. HE	LE(S) vs. HE	HE(C) vs. LE	HE(C) vs. LE
98	HE(S) vs. LE	HE(S) vs. LE	LE(B) vs. HE	LE(B) vs. HE	HE(B) vs. LE	HE(B) vs. LE	LE(T) vs. HE	LE(T) vs. HE
99	LE(C) vs. HE	LE(C) vs. HE	HE(T) vs. LE	HE(T) vs. LE	LE(S) vs. HE	LE(S) vs. HE	HE(B) vs. LE	HE(B) vs. LE

Table 4 Pair-wise comparisons of repellents in experiment 3 and the reference treatment chosen for calculation of preference score (intake of reference diet divided by total intake)

Comparison	Reference	Alternative
A	Blood	Thiram
B	Blood	Capsaicin
C	Capsaicin	Thiram
D	Sachet	Thiram
E	Sachet	Blood
F	Sachet	Capsaicin

each subject) using the Shapiro-Wilk test for normality (Proc Univariate; SAS 2002). The indifference response (0.5 minus HE preference score) was then subjected to *t*-test for the null hypotheis (mean=0) using the univariate procedure. Average total intake (sum of both diets) was determined for each subject, and the mean and standard error were calculated for later comparison with total intake during experiment 2.

Treatment preference scores were calculated for experiment 2 (treated diet intake divided by total intake). When total intake was zero (neither diet consumed), the preference score was considered a missing value. Fixed effects were: "protected" diet (either HE or LE receiving treatment); treatment (repellent); protect*treatment; position of the treated diet (right or left); protect*position; treatment*position; treatment*protect*position; and day. Separate ANOVA models also were produced for each level of protected diet (HE or LE) by using treatment, position, and treatment*position as fixed effects. Multiple comparisons of means were made by controlling the false discovery rate according to the procedures of Benjamini and Hochberg (1995). For HE diet protection, one *post-hoc* comparison of treatment*position was made for right and left positioning of the food container with sachet treatment. Total intake data also were subject to ANOVA with fixed effects: "protected" diet, treatment, protect*treatment, position, protect*position, treatment*position, treatment*protect*position, and day.

Four paired *t*-tests were conducted using data from experiment 1 and LE-protected diet data from experiment 2. Mean LE preference scores were calculated for each subject in experiment 1 (equal to 1—HE preference score as previously determined). Experiment 1 means were subtracted from experiment 2 preference scores according to subject. A *t*-test was conducted for each treatment using the univariate procedure in SAS. Each subject*day occurrence was considered a replicate for that treatment. False discovery rate for multiple comparisons was controlled by using the procedures of Benjamini and Hochberg (1995).

There were six pair-wise comparisons of repellents in experiment 3 (Table 3). Preference scores were calculated using one treatment as the reference (numerator) for all instances of that comparison. The indifference response (0.5 minus preference score) was calculated and subjected to ANOVA with comparison (Table 3), position of the reference treatment (right or left), comparison*position, and day the fixed effects. The null hypothesis (indifference response=0) was evaluated by *t*-test using the false discovery rate controlling procedure (Benjamini and Hochberg 1995).

Results

Experiment 1 Mean intake of the HE diet was 221±44 g and of the LE diet was 2.2±0.8 g. The resulting preference score (0.99) indicated a strong preference for HE diet ($P<0.001$). Preference scores were normally distributed ($P=0.625$) and not subject to day ($P=0.136$), position ($P=0.344$), or day*position ($P=0.852$) effects. Mean total intake for the ten subjects was 223±44 g.

Experiment 2 Preference scores were not subject to a day effect ($P=0.154$), but all other effects were highly significant ($P<0.001$). Inspection of treatment*protect*position means indicated that LE diet was avoided regardless of treatment or position, while avoidance of HE diets varied by treatment and position. Evaluation of protected LE diets alone confirmed that avoidance was not subject to treatment ($P=0.368$), position ($P=0.238$), or treatment*position ($P=0.587$). Treatment preference scores were less than 0.007 for all treatments (Fig. 1).

Paired *t*-tests indicated that two treatments reduced preference for LE diet in experiment 2 with respect to LE diet preference in experiment 1. Differences for capsaicin

Table 5 Assignment of pair-wise comparisons (Table 4) to the ten subjects in experiment 3. High energy (HE) diet was treated with test repellents and offered to the subjects in two-choice tests

Subject	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
5	D	D	B	B	C	C
21	E	E	C	C	D	D
22	D	D	F	F	A	A
25	B	B	D	D	E	E
92	A	A	F	F	C	C
93	C	C	E	E	F	F
95	B	B	C	C	E	E
97	F	F	A	A	B	B
98	A	A	D	D	B	B
99	E	E	A	A	F	F

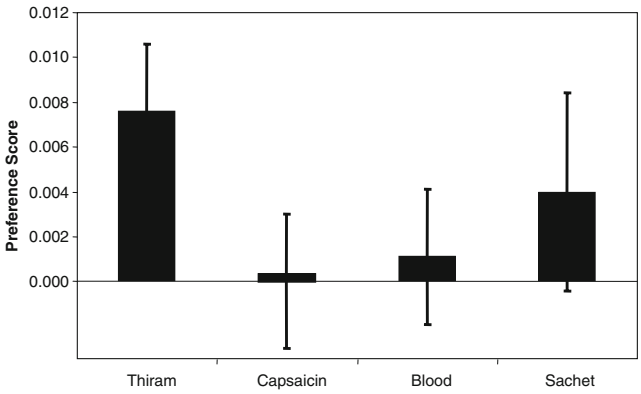

Fig. 1 LE diet preference scores (repellent-treated diet intake divided by total intake) did not differ significantly among repellent treatments (P=0.368). High energy (HE) diet was the alternative in 30 min two-choice tests. *Error bars* represent standard error

(−0.01; P=0.007) and blood (−0.009; P=0.022) reflected LE preference scores being reduced from approximately 0.01 in experiment 1 to nearly 0.0 in experiment 2 as a result of treating the LE diet with the test repellents (Fig. 2).

Conversely, avoidance of protected HE diets was subject to all fixed effects (P<0.001), including treatment*position. Thiram-treated HE diet was strongly preferred over the LE alternative, while both capsaicin and blood significantly reduced intake of the highly preferred HE diet regardless of position (Fig. 3). Interestingly, preference scores for HE diet presented on the left side with the sachet were higher than HE diet presented on the right side with the sachet (P< 0.001) while being consumed equally with the LE alternative.

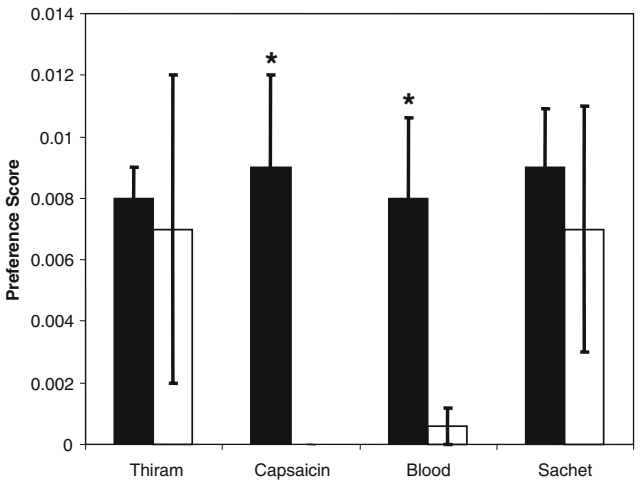

Fig. 2 Low energy (LE) preference scores (LE diet intake divided by total intake) from experiment 1 (pre-treatment intake in the absence of repellents) and experiment 2 (LE diets treated with repellent) in 30 min two-choice tests with high energy (HE) diet as the alternative. *Error bars* represent standard error. Paired *t*-tests indicated that preference scores differed significantly (*) between the experiments for capsaicin and blood (α=0.05). Legend: ■ Pretreatment (Experiment 1), □ Repellent (Experiment 2)

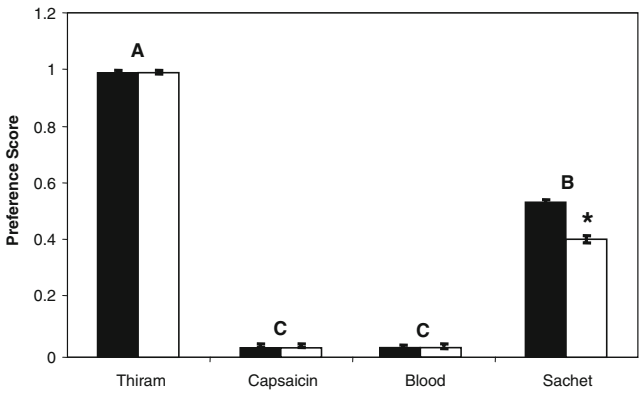

Fig. 3 Experiment 2 preference scores (repellent-treated diet intake divided by total intake) for high energy (HE) diets treated with the test repellents. Error bars represent standard error. Low energy (LE) diet was the alternative in 30 min two-choice tests. Letters indicate differences in preference scores due to repellent treatment (main effect). The asterisk indicates a position effect for one of the four treatments which led to the significant treatment*position interaction. Legend: ■ Treatment in left position, □ Treatment in right position

Total intake data was analyzed to determine if the odor of the test repellent influenced intake of both diets, not just the diet associated with the repellent. Total intake was impacted by diet protected (P=0.001), treatment (P= 0.003), and day (P=0.026). Total intake was 203±59.4 g when the LE diet was protected and 145±59.3 g when the HE was treated with repellent. This indicates that when HE diet was treated with effective repellents, subjects did not compensate for reduced HE intake with increased LE consumption. Treatment (P=0.003) and day (P=0.026) also were significant.

Among the treatments, total intake when one of the diets was treated with thiram (223±60.5 g) was not significantly different from capsaicin (189±60.5 g; Fig. 4). At the same time, total intake when blood (132±71 g), sachet (154± 60.5 g), or capsaicin treatments were applied to one of the

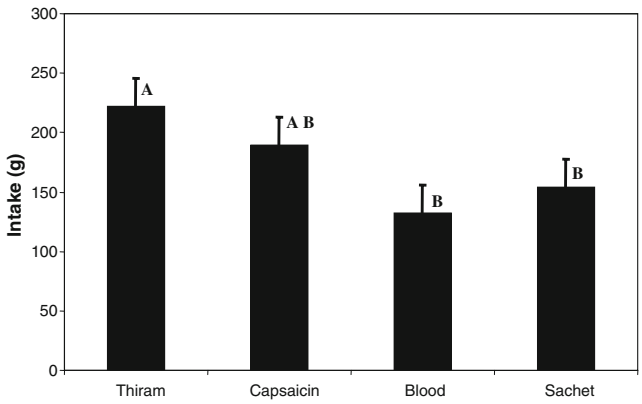

Fig. 4 Total intake data in experiment 2 of 30 min two-choice tests with either low energy (LE) or high energy (HE) diets treated with a repellent. Means marked with different letters are significantly different (α=0.05) and *error bars* represent standard error

diets were not statistically different. By comparison, total intake of HE and LE diet in the absence of a repellent (experiment 1) was 223±44 g.

Experiment 3 Treatment comparison was not subject to position (P=0.485), comparison*position (P=0.751), or day (P=0.745) effects. Further examination of the comparison effect (P<0.001) indicated that a preference for one of the repellents was evident for every two-choice comparisons except for capsaicin vs. blood (Fig. 5). Avoidance (repellency) of the treatments followed the order of greatest avoidance to least: blood = capsaicin > thiram > sachet.

Discussion

As intended, HE diet was significantly preferred by all subjects, by a factor of 99 to 1. By having a dramatic difference in preference for the two diets, subsequent experiments could be conducted in a manner that incorporated varying incentives to consume treated or alternative food items. Thus, experimental conditions modeled two extreme circumstances possible under field conditions: 1) the food source requiring protection from herbivory is highly desirable relative to the alternatives; and 2) alternative foods are more desirable than the food treated with repellents.

Deer strongly avoided repellent-treated LE diet when HE was available, regardless of repellent type (Fig. 1). Two repellent ingredients, capsaicin and blood, significantly reduced preference for the LE diet relative to untreated diets in experiment 1 (Fig. 2). Although statistically significant, the effect of capsaicin and blood avoidance was of no practical consequence, as LE preference scores were merely reduced from approximately 0.01 in the absence of repellents (experiment 1) to 0.0 when LE diets were treated with blood or capsaicin (experiment 2). When highly preferred alternatives are available, the less preferred food is easily protected.

A wider range of repellent activities were revealed when the highly desirable HE diet was treated with the repellents and the alternative was the less desirable (LE diet). In experiment 2, capsaicin and blood were extremely effective repellents when applied to the HE diet (Fig. 3). Previous studies have also shown that blood is an effective repellent (Nolte and Wagner 2000; Wagner and Nolte 2001; Kimball et al. 2008) and that avoidance of capsaicin is concentration dependent (Andelt et al. 1994). In the current study, subjects strongly avoided the HE diet treated with blood or 0.14% capsaicin despite being motivated to select the HE diet over the LE alternative.

Thiram was ineffective as a repellent. It was not unexpected for the deer to consume thiram-treated HE diet during the first two exposures to the treatment in experiment 2. In previous studies with mule deer (*Odocoileus hemionus*) and elk (*Cervus elaphus nelsoni*), Andelt et al. (1991, 1992) reported similar responses to thiram-treated foods. With both species, consumption of the thiram-treated food in cafeteria-type tests decreased daily in 4 day and 5 day experiments, as it took repeated exposures for subjects to develop an aversion to the familiar food. When familiar foods are treated with a toxin that promotes conditioned aversion, multiple exposures to the toxin-food pair are required to produce an avoidance response (Kimball and Nolte 2005). This is particularly true when the treatment does not impart a distinct cue (e.g., taste, visual, and/or odor). Unlike blood and capsaicin that discolored the diets, thiram treatment was not visually evident as the maximum absorbance of thiram

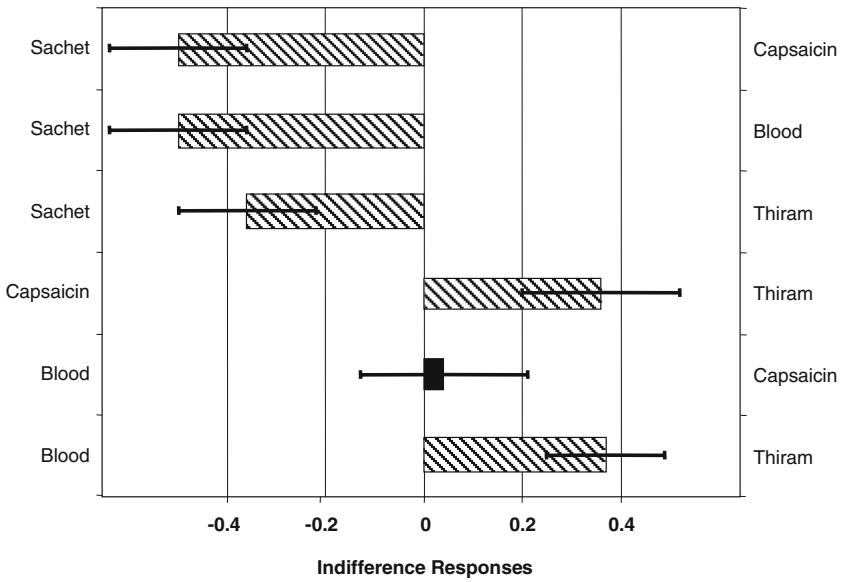

Fig. 5 Indifference responses from experiment 3 (with standard error bars). Indifference is indicated as a score of zero and determined by subtracting the preference score (intake of one choice divided by total intake) from the value of 0.5. Diagonal pattern indicates an indifference score significantly different from zero (α=0.05). The direction of the bar (positive or negative) indicates which of the two choices (right and left axes) was preferred in 30 min two-choice tests

(200–300 nm) is in the ultraviolet region (Talrose et al. 2009). Similar to many mammals, deer lack visual capability at ultraviolet wavelengths (Jacobs et al. 1994). Thiram is anecdotally reported to impart a bitter taste, but bitter taste is not a relevant cue to herbivores that forage in an environment replete with bitter plant stimuli (Nolte et al. 1994b).

The sachet was only moderately effective as a feeding deterrent in experiment 2 (Fig. 3). Several subjects were apprehensive about the sachet and limited consumption of diets placed in food bowls with attached sachets. However, there was tremendous variation among the subjects in response to the sachet treatment associated with HE diet. One subject ignored the sachet and readily consumed HE diet; one ate both diets; a few avoided the HE diet and consumed only LE diet; while others did not consume either diet. It was not evident why position (right or left presentation) of the sachet influenced repellency while position was not significant for the other repellents (Fig. 3). Odor may serve as a cue that can be associated with the palatability of foods via learning processes (Provenza 1995a). Learning about the odor of foods permits avoidance (or preference) at future encounters with that food on the basis of odor alone.

It is possible that the sachet was not the only treatment that served as a vapor repellent. Because volatiles were present throughout the test area (treatment odors were not confined to the treated diet), vapor repellents could be expected to influence intake of both diet choices. To test the effects of the treatments on intake of both choices, total intake data were analyzed for treatment and diet effects. Total intake data from experiment 2 suggests that non-volatile thiram had less influence on feeding from both food bowls than the more odiferous blood or sachet treatments (Fig. 4). Volatile components of blood treatment influenced not only intake of the treated diet, but also the alternative choice. Protein hydrolysis and lipid oxidation of animal-based stimuli (such as blood) produce volatile odors such as sulfides, aldehydes, and organic acids (Kamiya and Ose 1984). Sulfurous volatiles have been implicated in repellency of predator urine and egg (Nolte et al. 1994a; Lewison et al. 1995).

Volatile odors also may confer information regarding the surroundings. As such, certain odors such as blood, egg, meat, and bone meal have been thought to indicate danger from predators and have been called "fear" repellents (Nolte and Wagner 2000). However, behaviors such as approaches or head entries into feeders were unaffected by predator-based repellents (Pfister et al. 1990), and these repellents failed to exclude herbivores from treated locations as would be anticipated if predation were a consequence of foraging near the odor source (Belant et al. 1998; Nolte and Wagner 2000). Predator odors may provide cues regarding predator density and influence decisions about where and when to forage, but do not influence intake during a feeding bout once the decision to forage has been made (Chabot et al. 1996).

Extinction (cessation of avoidance behavior) among repellents that do not produce pain or malaise is likely to occur when the treated food is highly desirable. When the basal ration was restricted, deer and elk increased daily consumption of food treated with egg product (Andelt et al. 1991, 1992). In a winter field study conducted when alternative foods were scarce, plants treated with an egg-based repellent and netting were not protected (Milunas et al. 1994). At the same time, reappearance of alternative food sources can result in recurrence of the avoidance behavior. In a previous study, addition of casein to a ground diet reduced its intake relative to the unadulterated diet (Kimball et al. 2005). When the casein diet was offered subsequently to deer in a single-choice experiment, avoidance was not observed. Yet, after readily consuming the casein diet for 8 days, significant avoidance of casein-treated diet was evident when offered again in the presence of the unadulterated diet. Therefore, the mechanism by which repellency is achieved with animal-based products is not neophobia.

Avoidance of blood and other animal-derived substances may be the result of an "evolutionary memory" (Provenza 1995b) that conveys information about potential sources of pathogens. Just as a wide variety of toxins have been associated with bitter taste to humans (Bachmanov and Beauchamp 2007), compounds indicative of pathogenic activity (e.g., certain proteins or peptides) may be distinctly identified by herbivores. Importantly, the food item must be "contaminated" with these compounds in order to affect intake. Animal products employed as vapor repellents may alert foraging herbivores about the potential of contamination, and reduce their intake until habituation occurs (e.g., sachet). However, significant repellent efficacy is achieved when the animal product is in contact with the food and the herbivore avoids the treated food thus avoiding potential pathogens (e.g., blood).

In contrast to experiment 2, fewer subjects avoided the sachet in experiment 3; while avoidance of thiram treated HE diet was more evident (Fig. 5). Although not specifically tested in this study, experience with the sachet during experiment 2 may have contributed to habituation to the odors. Similarly, experience with thiram-treated diets also may have facilitated learning about the postingestive effects of thiram. Animals can learn that novel cues are associated with positive consequences (or have no associated consequences) as well as they can learn about negative consequences (Provenza 1995b). These data suggest that learning was not a necessary component of blood and capsaicin avoidance. When highly desirable diets were treated with blood or capsaicin (experiment 2), as well as

in head-to-head comparisons (experiment 3), the flavor modification (blood) and irritation (capsaicin) mechanisms continued to demonstrate significant repellency (Fig. 5).

In field applications, existence of alternative forage options is not always evident. It may not be feasible to compare nutritional quality of the agriculture resource in need of protection with forage alternatives. However, it is clear that when valuable resources are at risk, effective tools are needed to minimize losses due to herbivory. The results of our study suggest that repellents that rely only on neophobia as the mode of action may be effective in field applications only when foraging options are plentiful. Furthermore, repellents that rely on conditioned aversion may not be useful. The deer in this study did not reduce intake upon initial offering of thiram-treated diets. Thiram may be an impractical repellent if numerous exposures are required to condition an aversion. Conversely, repellents with immediate consequences to the consumer (i.e., irritation and flavor modification) can be effective repellents, even when the treated resource is highly desirable in comparison to other foraging options.

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Allelochemicals can induce a wave of ROS and a Ca^{2+} signaling cascade, with subsequent genome-wide changes in gene expression and root death in plants (Bais et al. 2003; Ding et al. 2007). Some factors involved in the signaling transduction pathway have been characterized (Baerson et al. 2005; Golisz et al. 2008). All physiological, biochemical, and transcriptional changes are implicated directly or indirectly in plant growth inhibition.

We are interested in phytotoxic phenomena in cucurbit plants, which exhibit great genetic variation in autotoxic potential (Yu et al. 2000). Similar to root exudates of asparagus and other plants, benzoic and cinnamic acids are found in root exudates of cucumber (Young 1984; Yu and Matsui 1994; Wu et al. 2000). They produce detrimental effects on ion uptake, and enhance the incidence of Fusarium wilt by causing oxidative stress in roots (Yu and Matsui 1994, 1997; Ye et al. 2004, 2006). Interestingly, root exudates of both cucumber and watermelon show high autotoxicity, but not toxicity to other species such as figleaf gourd (Yu et al. 2000, 2003). As *trans*-Cinnamic acid (CA) is a principal compound in root exudates and plant extracts of cucumber and other species, it often has been studied (Yu and Matsui 1994, 1997; Chon et al. 2003; Fujita and Kubo 2003; Ye et al. 2004, 2006; Blum 2005; Hiradate et al. 2005). *cis*-Cinnamic acid has been found in *Brassica parachinensis* and *Arabidopsis thaliana*, but the biosynthesis is not well understood (Yin et al. 2003; Wong et al. 2005). We have demonstrated that exposure to *trans*-CA significantly increases ROS generation and accumulation in cucumber roots but has minimal effect on ROS metabolism in figleaf gourd (Ding et al. 2007). Here, we investigated whether the different growth responses to *trans*-CA in cucumber and figleaf gourd are associated with a difference in uptake. We examined further whether Ca^{2+} signaling was involved in the interspecies recognition.

Methods and Materials

Plant Material and Growth Bioassay Cucumber (*Cucumis sativus* L. cv Jinyan No.4) and figleaf gourd (*Cucurbita ficifolia* Bouché) seeds were surface-sterilized in 1% (v/v) NaOCl for 5 min, rinsed thoroughly with deionized H_2O, and germinated on filter paper saturated with 0.5 mM $CaSO_4$ at 28°C for 6 d in the dark. Germinated seeds were exposed to light for 10 h and subsequently transferred to Enshi nutrient solution at pH 6.5 and electrical conductivity at 1.2 ms cm^{-1} (Yu and Matsui 1994). Then, they were maintained at a relative humidity of 95–100%, a photosynthetic photon flux density (PPFD) of 500 μmol m^{-2} s^{-1}, and a temperature of 25–30°C for 8 d.

Each of the 15-d-old seedlings then was incubated in 25 ml Enshi nutrient solutions in beakers containing 0 mM, 0.05 mM, 0.10 mM, or 0.25 mM *trans*-cinnamic acid (CA). The beakers were wrapped with aluminium foil to prevent the isomerization between *trans*- and *cis*-CA. *trans*-CA was dissolved in EtOH, and the final concentration of EtOH in each solution including the control was 0.1% (v/v), at which concentration EtOH has a negligible effect on cucumber plants (Yu and Matsui 1997).

Each treatment had eight plants and was done in triplicate. Five days later, plants were harvested, oven dried at 80°C for 3 d, and weighed. Relative growth inhibition (RGI) was calculated by $[(W_t-W_0)/W_0]/100$. W_t and W_0 were dry weights for seedlings after exposure to the *trans*-CA treatments and unexposed controls, respectively.

In Vivo trans-Cinnamic Acid Uptake by Roots Roots of 20-d-old seedlings of both species were excised and placed into an aerated medium consisting of 25 mM MES, 25 mM Tris, 0.5 mM KCl, 0.25 mM $CaSO_4$, and 0.1% sodium ascorbate with or without 0.25 mM *trans*-CA (Schulz et al. 1993). The uptake of *trans*-CA was determined indirectly by its decrease in the incubation solution. Free *trans*-CA in the incubation solution and in the roots were extracted as described by Schulz et al. (1993) and determined by HPLC (Shimadzu LC-10AT, Japan) using a C_{18} column with UV detection at 280 nm and a flow rate of 1.0 ml min^{-1}.

Gene Transcription and Metabolite Analysis To determine changes in the transcript of cinnamate 4-hydroxylase (C4H), a gene that encodes the enzyme that catalyzes the biosynthesis of *p*-coumaric acid from *trans*-CA, the roots of cucumber plants were sampled at 0 h, 4 h, 8 h, 12 h, and 24 h after inoculation. Total RNA was extracted with Trizol (Sangon, China) and reverse-transcribed with olig (dT) using reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Specific primers for quantitative real time PCR (qRT-PCR) were designed in function of its conserved sequences: forward primer 5′-CTCACATGAACCTCCACGAC-3′ and reverse primer 5′-CCTCTTCCAAGAACCTCTCG-3′. qRT-PCR was performed using the iCycler iQTM Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA). The PCR conditions consisted of denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. A dissociation curve was generated at the end of each PCR cycle to verify that a single product was amplified by using software provided with the Real-time PCR Detection System. Identity of the PCR products was verified by single strand sequencing using MegaBACE 1000 DNA analysis system (Amersham Biosciences, USA).

Four glycosidases (all purchased from Sigma, USA) were tested for enzymatic cleavage of the metabolites. These glycosidases include a glucosidase (from *Aspergillus niger*), an α-glucosidase (from *Saccharomyces cerevisiae*),

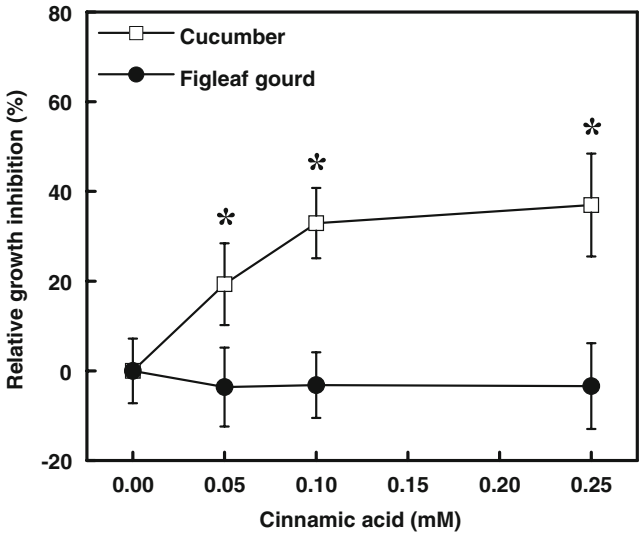

Fig. 1 Effects of *trans*-cinnamic acid (CA) (1) on root growth of cucumber (*open squares*) and figleaf gourd (*solid circles*) plants. Samples were taken 3 d after treatment. Each treatment involved eight plants and was done in triplicate. Data are represented as the means of three independent replicates with standard error bars. *Asterisks* indicate a significant difference between the untreated control and the *trans*-CA treatment within the species by Student *t*-test ($P \leq 0.05$)

a β-glucosidase (from almonds), and an esterase (from rabbit liver). The assays were carried out as described by Schulz et al. (1993). Reaction mixtures were analyzed for the decreases of the metabolite peaks and the presence of *trans*-CA, *p*-coumaric acid and ferulic acid by HPLC.

Fixation and Transmission Electron Microscopy of Roots After exposure to 0.25 mM *trans*-CA for 10 h, the 5-d-old seedling roots were fixed in 3% glutaraldehyde, 2% potassium pyroantimonate, and 75 mM potassium phosphate (pH 7.8) at 4°C for 4 h, and washed four to five times in 75 mM potassium phosphate and 2% potassium pyroantimonate (pH 7.8) every 30 min. Samples subsequently were fixed in 1% OsO_4 containing 2% potassium pyroantimonate and 75 mM potassium phosphate (pH 7.8) for 12–16 h at 4°C, rinsed in 60 mM potassium phosphate buffer four to five times (30 min each time), dehydrated in a graded EtOH series, and embedded in Epon 812 resin (Qin et al. 2005). Ultrathin sections were obtained using a Sorvall MT-6000 ultramicrotome (Wilmington, Germany), stained with 2% uranyl acetate for 15 min, and observed under a JEM-1200 EX transmission electron microscope (TEM Hitachi, Japan).

Loading of Fluo-3/acetoxymethyl Ester into Root Cells and Confocal Laser Scanning Microscopy A Zeiss Axioskop 2 epifluorescence LSM 510 confocal microscope (Carl Zeiss Mikroskopie, Jena, Germany) was used to determine $[Ca^{2+}]_{cyt}$ levels. Intact roots (1 cm to 2 cm long) of 7-d-old seedlings of both species were incubated in a solution containing 17 μM fluo-3/acetoxymethyl ester, 50 mM sorbitol, and 0.2 mM $CaCl_2$ at 4°C for 0.5 h in the dark. The dye-loaded roots were incubated in an aerated 0.2 mM $CaCl_2$ solution with or without 0.25 mM *trans*-CA at 25°C for 0.5 h in the dark (Zhang et al. 1998). Intact roots were mounted in a Plexiglas chamber (2 ml) with a clean cover slip attached to the bottom, and flushed with a control solution for about 10 min prior to confocal imaging with a Zeiss Axioskop 2 epifluorescence LSM 510 confocal microscope fitted with a Spot CCD camera (Diagnostic Instruments,

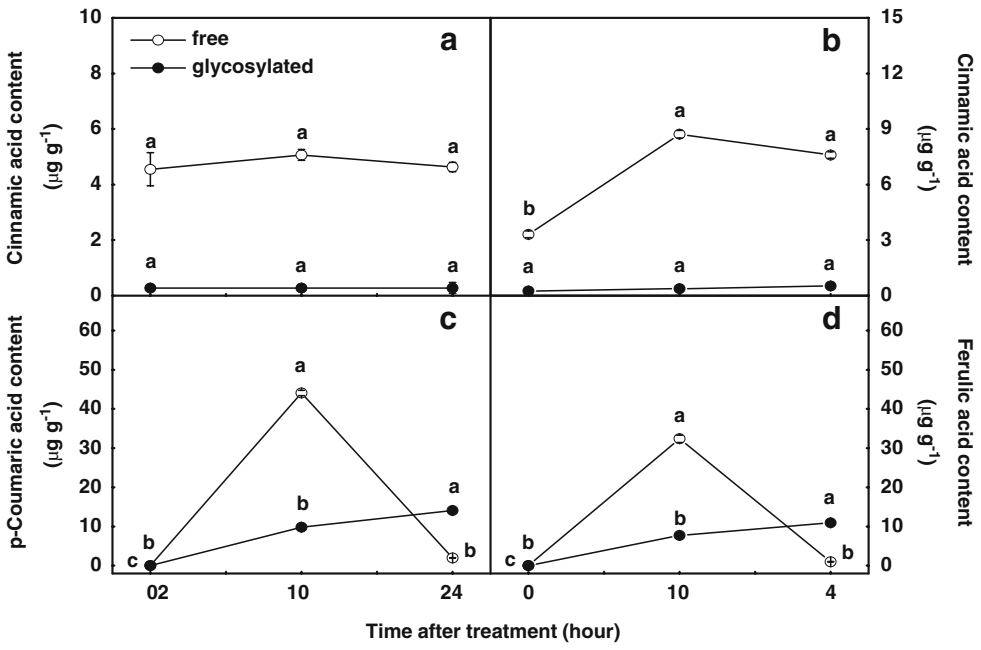

Fig. 2 Changes in free *trans*-cinnamic acids (*open circle*, CA) and glycosylated cinnamic acids (*solid circle*) concentrations in cucumber and figleaf gourd roots after exposure to 0.25 mM *trans*-CA. Data are the mean of three experiments with standard error bars. **a** figleaf gourd roots; **b**, **c**, and **d**, cucumber roots. Different letters indicate a significant difference between the untreated control and *trans*-CA treatment over time within the species by Student *t*-test ($P \leq 0.05$)

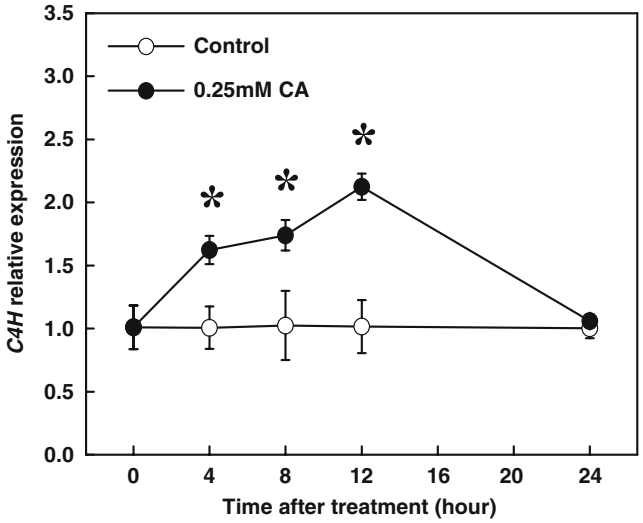

Fig. 3 Changes in C4H transcript level in roots of cucumber after exposure to 0.25 mM *trans*-cinnamic acid (CA). Data are the mean of three experiments with standard error bars. *Asterisks* indicate a significant difference between the untreated control and *trans*-CA treatment over time y Student *t*-test ($P \leq 0.05$)

Sterling Heights, MI, USA) with 488 nm excitation and 515 nm emission. The same apical cells of six to eight roots of each treatment were scanned consecutively every 5 min for up to 30 min. The intensity of green fluorescence was analyzed by LSM 5 image software.

Observation of Cell Viability Viability of root tips was determined by staining with fluorescein diacetate (FDA), as described by Ishikawa and Wagatsuma (1998). In brief, roots were stained with FDA (12.5 μg ml^{-1}) for 10 min, then observed under a fluorescent microscope with light excitation (488 nm), and photographed (Leica DMLB). The same apical cells of six to eight roots of each treatment were scanned consecutively every 5 min for up to 30 min.

Results and Discussion

Selective Growth Inhibition The inhibitory effect of *trans*-cinnamic acid (CA) (Fig. 1) on plant growth was specific and concentration-dependent. Exposure to 0.05 mM *trans*-CA resulted in a significant inhibition of cucumber root growth. At a concentration of 0.25 mM, *trans*-CA inhibited root growth by 36.97%. In contrast, it had little effect on figleaf gourd growth, even at the highest concentration. This result supported earlier findings that *trans*-CA inhibited plant growth of cucumber but not figleaf gourd (Yu et al. 2000; Ding et al. 2007). Although the role of *trans*-CA under field conditions remains to be determined, it has been found in root exudates or root residues from several plants that exhibit high phytotoxicity to seed germination and seedling growth (Young 1984; Yu and Matsui 1994; Wu et al. 2000; Hiradate et al. 2005; Wong et al. 2005). *cis*-CA has been found in several plant species, and it exhibits much more phytotoxicity than *trans*-CA (Yin et al. 2003; Hiradate et al. 2005;Wong et al. 2005). Importantly, *trans*-CA is easily isomerized into *cis*-CA in the presence of sunlight or UV light (Wong et al. 2005). In this study, light was completely kept from the incubation solution, and isomerization between CA isomers could not occur in the incubation solution or the roots. Accordingly, the phytotoxicity to cucumber roots could be attributed to the direct effects of *trans*-CA.

Reasons for selective inhibition of plant growth remain elusive, although autointoxication has been suggested as a protective mechanism that prevents future conspecific competition for both annual and perennial plants (Singh et al. 1999; Canals et al. 2005). It seems probable that all known mechanisms of transport of substances through cell membranes participate in the translocation into root space, and from there, into the environment.

trans-Cinnamic Acid Uptake and Associated Metabolism To determine whether the different growth responses are associated with a difference in *trans*-CA uptake, changes

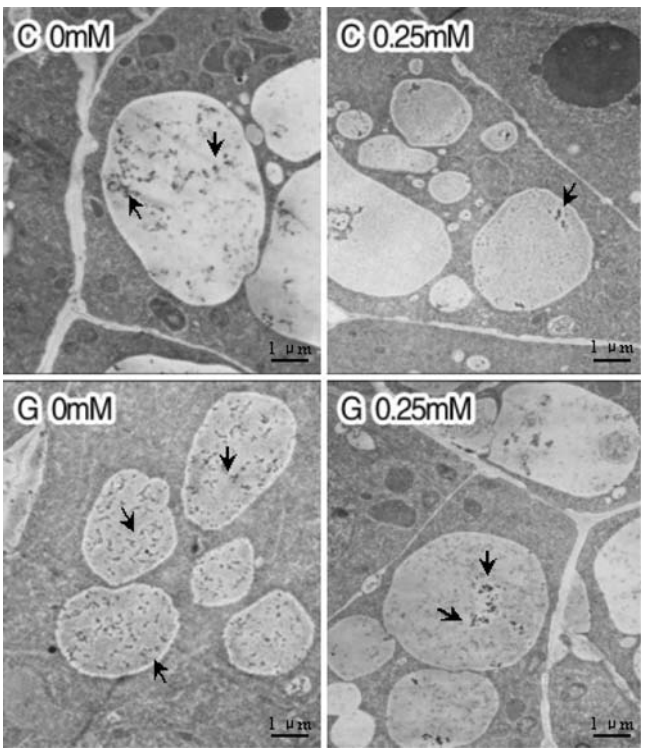

Fig. 4 Localization of calcium in root cells of cucumber and figleaf gourd exposed to 0.25 mM *trans*-cinnamic acid (CA). C 0 mM, cucumber control roots; C 0.25 mM, cucumber roots exposed to 0.25 mM *trans*-CA for 3 h; G 0 mM, figleaf gourd control roots; G 0.25 mM, figleaf gourd roots exposed to 0.25 mM *trans*-CA for 3 h. Photos were taken at ×6000 magnification. *Arrows* show calcium precipitates by dye

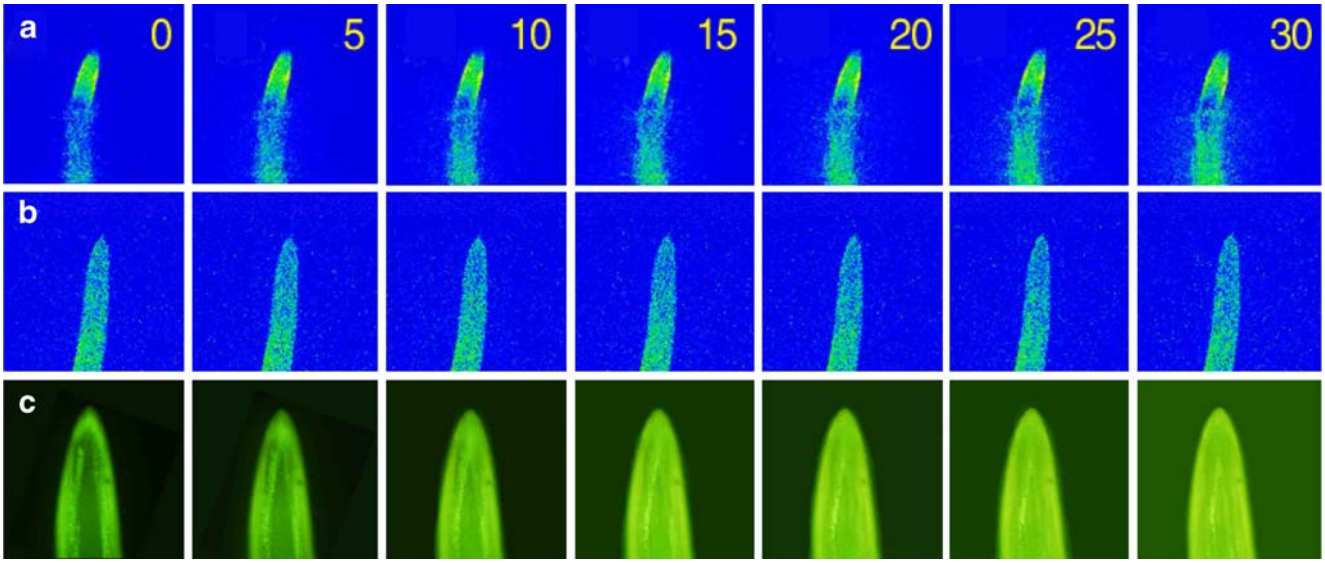

Fig. 5 Changes in $[Ca^{2+}]_{cyt}$ level and cell viability for roots of cucumber and figleaf gourd after exposure to 0.25 mM *trans*-cinnamic acid (CA). A), $[Ca^{2+}]_{cyt}$ level in cucumber roots; B), $[Ca^{2+}]_{cyt}$ level in figleaf gourd roots; C), cell viability of cucumber roots. Figures represent minutes after the *trans*-CA treatment. Photos were taken at ×5 magnification for Fig. 5a, b and ×10 magnification for 5c. Note: (1) Green fluorescence intensity increases indicate increased $[Ca^{2+}]_{cyt}$ level in Fig. 5a, b; (2) *Green color* indicates live cells while *yellow colour* indicates dying cells in Fig. 5c. High green fluorescence indicates high cell viability, while decreased fluorescence indicates leakage of dye from dead cells

in *trans*-CA concentrations in the incubation solution and in roots of these two species were determined. *trans*-CA concentrations remained 0.25 mM in the incubation solution with figleaf gourd roots, while it decreased from 0.25 mM to 0.05 mM in the cucumber root solution after 24 h incubation (data not shown). Thus, ca.73.2% of *trans*-CA in the solution was taken up by cucumber roots but little by figleaf gourd roots. Consistent with this, no significant change in free *trans*-CA concentration was found in roots of figleaf gourd (Fig. 2a). In comparison, free *trans*-CA in cucumber roots increased from 3.2 μg g^{-1} to 8.72 μg g^{-1} and to 7.59 μg g^{-1} at 10 h and 24 h after incubation, respectively (Fig. 2b). Similarly, significant increases in free *p*-coumaric and ferulic acid concentrations were observed at 10 h, but not at 24 h (Fig. 2c, d). In addition to free CAs, conjugated CAs also exist (Funk and Brodelius 1990). Of the four glucosidases and esterase tested, only β-glucosidase from almonds released the conjugated CAs, thus leading to increased amounts of free CAs, and suggesting that glycosylated CAs were present as metabolites after exposure to *trans*-CA, as found by Schulz et al. (1993). However, glycosylated *trans*-CA concentration was very low, and little increase was observed in *trans*-CA-incubated cucumber and figleaf gourd roots. In comparison, there were significant increases in the concentrations of glycosylated *p*-coumaric and ferulic acids in *trans*-CA-incubated cucumber roots (Fig. 2).

These findings are in agreement with results obtained in *Vanilla planifolia*, in which *trans*-CA glucosyltransferase preferentially accepts *p*-coumaric and ferulic acid as substrates, while *trans*-CA is a poorer substrate (Funk and Brodelius 1990). Modified phytochemicals such as glycosylated conjugates through the activity of glycosyltransferases are commonly deposited in vacuoles (Schulz and Friebe 1999). Furthermore, exposure of cucumber roots to *trans*-CA significantly increased transcripts of *C4H* (cinnamate 4-hydroxylase), a gene encoding the enzyme that

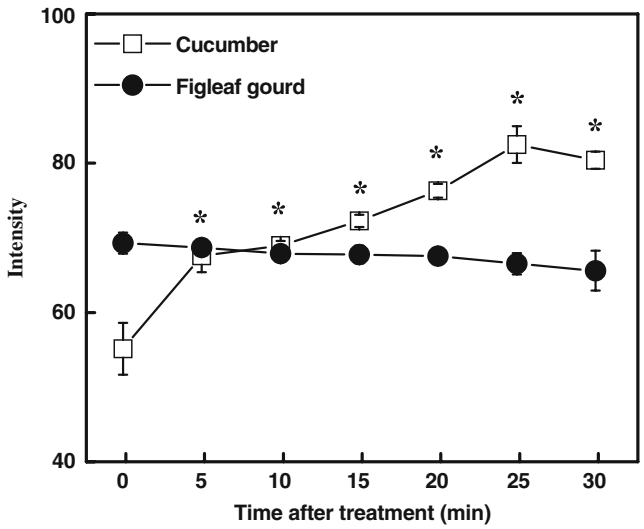

Fig. 6 Changes in $[Ca^{2+}]_{cyt}$ intensity in the cells of cucumber (*open square*) and figleaf gourd (*closed circle*) roots after exposure to 0.25 mM *trans*-cinnamic acid (CA) (photos shown in Fig. 5a and b). Data are the means of three experiments with standard error bars. *Asterisks* indicate a significant difference between the untreated control and the *trans*-CA treatment over time within the species by Student *t*-test ($P \leq 0.05$)

catalyzes the biosynthesis of *p*-coumaric acid from *trans*-CA (Fig. 3). Accordingly, selective *trans*-CA uptake appears to be the mechanism responsible for the differences in growth response of the two species, and the conversion to other CAs associated with glycosylation might be an important detoxification mechanism for *trans*-CA in cucumber.

Ca^{2+} Release and Cell Viability Calcium plays a crucial role in plant growth and development, e.g., acting as a second messenger in signal transduction (Hetherington and Brownlee 2004; Lecourieux et al. 2006). It is present in three forms: bound, free, or stored, and is distributed in the apoplasm, the cytoplasm, and intracellular organelles (endoplasmic reticulum and vacuoles). Transient spatial and temporal changes in calcium pools and cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) are initial responses to external stimulation, which may trigger a physiological and developmental cascade (Knight 2000; Lecourieux et al. 2006). Transmission electron microscopy analysis demonstrated that calcium precipitates were distributed primarily in vacuoles of cucumber and figleaf gourd root cells (Fig. 4). These precipitates disappeared after treatment with EGTA (data not shown), suggesting that they were calcium antimonite deposits. Intriguingly, vacuole calcium precipitates were decreased greatly in cucumber root cells after *trans*-CA treatment, while almost no effect was observed in figleaf gourd root cells. This indicates that the vacuole-stored calcium was released in cucumber root cells upon exposure to *trans*-CA.

A pivotal role for cytosolic free Ca^{2+} ions revolves around its action as a second messenger in the transduction of hormonal and environmental signals (Lecourieux et al. 2006). Salinity, drought, oxidative stress, and osmotic shock all induce an increase in $[Ca^{2+}]_{cyt}$ resulting from both extracellular Ca^{2+} influx and intracellular Ca^{2+} release (Knight et al. 1997; Pandey et al. 2000). To determine whether Ca^{2+} fluctuation in the vacuole is associated with a difference in cytoplasmic Ca^{2+} concentration, the $[Ca^{2+}]_{cyt}$ was imaged after roots were loaded with fluo-3/acetoxymethyl aster, a fluorescent Ca^{2+}-sensitive probe (Fig. 5a and b). After *trans*-CA treatment for 30 min, the fluorescence intensity of $[Ca^{2+}]_{cyt}$ dye was increased by 45.84% in cucumber (Fig. 6). By contrast, the root $[Ca^{2+}]_{cyt}$ level was unchanged in figleaf gourd. A significant $[Ca^{2+}]_{cyt}$ change was found in the elongation zone at the cucumber root tip. Moreover, *trans*-CA treatment induced a gradual reduction of green fluorescence in cucumber root-top cells, suggesting that these cells were losing viability during exposure to *trans*-CA treatment (Fig. 5c).

The transient $[Ca^{2+}]_{cyt}$ elevation in cucumber suggested that Ca^{2+} signaling was involved in the phytotoxic response in susceptible species. Our data are consistent with an earlier observation in which an aluminium (Al)-induced $[Ca^{2+}]_{cyt}$ increase was greater in an Al-sensitive wheat line than in an Al-tolerant line, and increases in $[Ca^{2+}]_{cyt}$ were correlated with root growth inhibition (Zhang and Rengel 1999). Disturbance of cytoplasmic Ca^{2+} homeostasis and subsequent breakdown of Ca^{2+}-mediated signal transduction have been suggested as a primary mechanism for Al rhizotoxicity in plants (Zhang and Rengel 1999). There could be, however, various mechanisms that would come into play in post Ca^{2+} release in the cytosol. One likely probability would be a change in cytosolic pH (Bais et al. 2003). Accordingly, further work is necessary on the relationship between *trans*-CA-induced loss of Ca^{2+}-homeostasis and impaired root growth before we can conclude the role of Ca^{2+}-mediated signaling in phytotoxicity.

In summary, we demonstrated that selective *trans*-CA uptake in cucumber roots induced an intracellular release of Ca^{2+} from vacuoles to cytoplasm, leading to a $[Ca^{2+}]_{cyt}$ increase in cucumber roots. The $[Ca^{2+}]_{cyt}$ increases in root apical cells corresponded well with the inhibition of root growth. Taken together, our results suggest that disturbance of $[Ca^{2+}]_{cyt}$ homeostasis often might be involved in the signaling response to allelochmicals.

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